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INTRODUCTION TO THE BACTERIA

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# *Introduction to the Bacteria*

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Stanford University*

SECOND EDITION

INTERNATIONAL STUDENT EDITION

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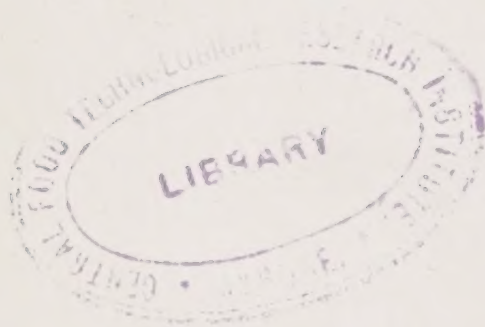
## INTRODUCTION TO THE BACTERIA

INTERNATIONAL STUDENT EDITION

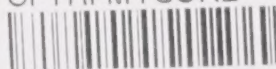
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Dedicated to my wife and children  
and to those students who develop an interest  
in the bacteria, as bacteria





## PREFACE

It is only natural, man's curiosity being what it is, that he attempts to learn something concerning the nature of life and of other organisms around him. Many studies have centered on the bacteria, forms of life so small as to be barely visible under the microscope yet at the same time capable of producing extensive changes in their environment, changes either beneficial or harmful to man. Information concerning the nature and activities of the bacteria and related organisms is so voluminous that no attempt is made to summarize it in this introduction to the bacteria, but instead the field of bacteriology is surveyed in such a manner as to introduce the student not only to descriptive bacteriology but also to the modern concepts of the how and the why of bacterial behavior. The presentation necessitates the omission of much descriptive matter concerning individual families of bacteria, this material being illustrated by a brief consideration of only one family, the Enterobacteriaceae, and its more commonly studied constituent species. Additional information regarding individual species can be obtained in the laboratory or in reference works.

Bacteriology has progressed from a primarily descriptive science to one in which serious attempts are being made to interpret the bacteria and their behavior on the basis of modern biological, physical, and chemical concepts. In particular, in recent years the application of biochemical principles to the study of the bacteria has considerably enriched our understanding of these organisms. This book is the result of endeavors over many years to digest and to condense for the general student the concepts of the what, the how, and the why of bacteria and bacterial activity in as simple and brief a presentation as appears to be consistent with the development of a fundamental understanding of the bacteria and related forms of life. Such a presentation involves biochemistry to an extent which at times may appear beyond the grasp of the general student; yet the author has found that the student can understand such concepts as the reactions involved in alcoholic fermentation if they are presented as an orderly mechanism involved in the respiratory activity of many forms of life, and not as a series of reactions and formulas to be memorized.

The contents of Chapter 8 are intended to serve as a general introduction to the metabolic activities of the bacteria and related microorganisms. This simplified treatment of a complex subject could be sufficient for an introductory course in general bacteriology. Chapter 9, devoted to mechanisms of respiration, is presented primarily as reference material. The somewhat advanced biochemical treatment employed in the chapter is designed to give the student a more fundamental picture of the chemical activities of the bacteria. As much, or as little, of this material can be employed by the instructor as he deems essential for the development of his plan or presentation. Omission of Chapter 9 as required reading matter would not seriously handicap the development of the subject matter which follows. In either the simplified or the more complex plan of treatment of metabolism employed in this text, as well as in other considerations of the bacteria in the remaining chapters, emphasis is placed on principles rather than on details concerning the bacteria and their behavior.

Once an understanding of the general principles of bacteriology has been developed, the student who is interested in any of the diverse fields of this science can utilize the more detailed descriptions of species, genera, and higher groups found in the larger general or specialized texts, monographs, and review articles. On the other hand, the general student can become familiar with the bacteria and their activities without being burdened by a welter of detail important to the specialist. Bacteriology becomes a dynamic rather than a static science with concepts subject to change as data accumulate, or as old data are reinterpreted on the basis of advances in our thinking. Unsettled problems, as well as the established ones, are presented from different viewpoints when possible to illustrate the dynamic nature of modern bacteriology. It is well for the student to realize from the start that not only do the bacteria vary but also man's concepts of the bacteria and their behavior are subject to change. Variations also exist in individual interpretations of the phenomena presented by these microbial forms.

I wish to thank my colleagues for their assistance, criticism, and advice and at the same time to relieve them of any responsibility for the final selection of the material presented and the manner of presentation. My apologies and thanks are extended to former students who served as "guinea pigs" in the development of the latter. My thanks are also extended to those numerous workers who have been involved in the collection and interpretation of information presented herein and to the various authors, publishing houses, and commercial firms who have so generously made available to me illustrations of bacteria and other matter pertinent to a study of the bacteria and their behavior. Reference is given to the source of each borrowed illustration in its accompanying

legend. The bibliography is limited primarily to recent original articles and to reviews on particular topics or groups of bacteria. This book has been thoroughly revised and corrected where necessary to bring it up to date. Obsolete material has been deleted and additional topics are included to illustrate aspects of microbial activity not covered in the first edition.

*C. E. Clifton*





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## CHAPTER 1

### INTRODUCTION TO THE BACTERIA

On all sides, microbes, or microorganisms, surround us and make felt their influence for good or evil. Some are benefactors, and without their activities in nature, life as we know it could not exist; others are agents of infection and death. Microbes (*micro*s, small; *bios*, life) may be defined as forms of life too small to be readily visible with the naked eye whose distinguishing characteristics become evident only with the aid of a microscope. Widely diverse organisms are included under the general term microorganisms; some of these are plants, others animals; some are composed of a number of cells, others are unicellular forms of life. They differ greatly in structure and in mode of life, their main common characteristics being their small size and apparently simple structure.

Microorganisms vary markedly in size from species to species; on one side we find microbes such as the molds which are visible to the eye; at the other extreme, cells so small as to be resolved only with difficulty under the best microscopes. At the lower limits it is difficult to ascertain the border line between animate and inanimate matter, at the upper limits between microbes and the higher forms of life. However, we shall find that many other properties used in describing microorganisms can be no more accurately defined. Bacteria, the microorganisms which we will consider in most detail, are near the lower limits of microscopic visibility.

Various bacteria were observed as early as 1676, but actually the science of bacteriology had its beginnings only about ninety years ago. Before 1860 bacteria were mainly of interest to microscopists and to those who cared to speculate about the nature of these small forms of life. One important question in this period of speculation concerned the origin of bacteria and other microbes: Did they always develop from their own kind or could they arise spontaneously? Experimental methods were crude, and reliable observations few and far between, but the controversies they engendered stimulated interest in the microbial world. It became a field fertile for study, and in the period around 1860 to 1880 the foundations of the science were laid by the great French scientist Louis Pasteur in his studies on the activities of bacteria. With awakening interest in the study of bacteria, of what they do and what they are,

more reliable information became available, techniques were developed and improved, particularly by the German school headed by Robert Koch, and around 1880 bacteriology entered the modern period of growth and development. It emerged as a science in its own right with interests connected with all aspects of our own life and of the animate and inanimate world upon which we are dependent. Let us trace the early development of this science before we proceed to a consideration of the bacteria as such.

**The Discovery of Bacteria.** During the latter part of the seventeenth century there lived in Delft, the Netherlands, an inquiring amateur scientist, Anthony van Leeuwenhoek. In his day it was not yet entirely safe to be too inquisitive about the life that surrounds us, yet in his spare time Leeuwenhoek ground lenses and constructed simple microscopes with which to pursue his hobby, the observation of the structure and form of both living and dead matter. His microscopes were crude compared with modern instruments, but they yielded clear images with sufficient magnification to unfold to his observing eyes a new universe.

Under his microscopes Leeuwenhoek observed the structure of striated



FIG. 1-1. Anthony van Leeuwenhoek, the father of bacteriology



muscle, of nerves, and of the crystalline lens and retina of the eye; he observed the spermatozoa, the red blood corpuscles and their circulation in the capillaries, and a variety of microorganisms. A number of microorganisms had been observed prior to the observations of Leeuwenhoek, but he saw and accurately described for the first time numerous forms of microbial life. His insatiable curiosity led him to continue his observa-

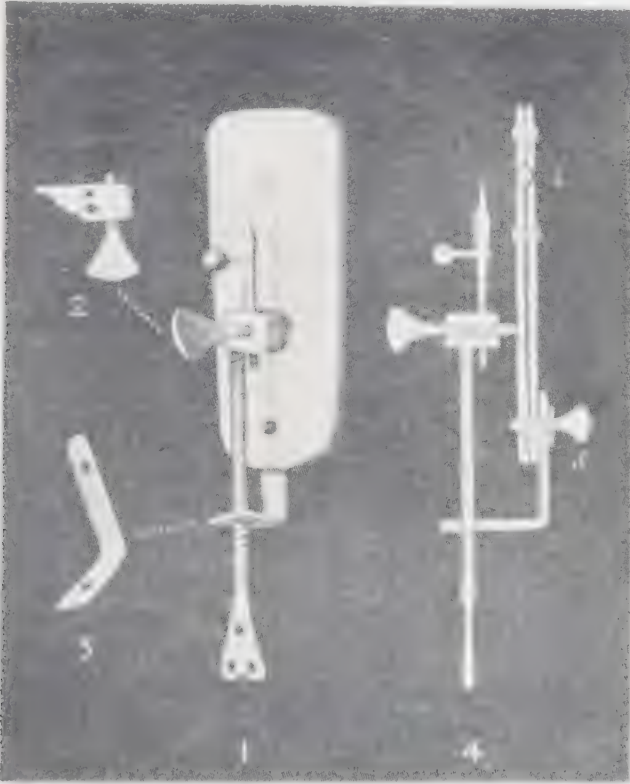


FIG. 1-2. Diagram of a Leeuwenhoek microscope. (From C. Dobell, "*Antony van Leeuwenhoek and His 'Little Animals.'*" John Bale, Sons and Danielsson, Ltd., London, 1932.)

tions on all things surrounding him, and in a long, gossipy letter to the Royal Society of London in 1676 he described, along with other microbes, *animalcules* or *wee small beasties* we now recognize as bacteria.

One day he was examining pepper grains in the hope of finding what imparted the biting taste to pepper, but without success. To render the grains easier to handle, he soaked them in water and later observed a drop of this pepper infusion under the microscope. It was teeming with wee forms, smaller than he had ever seen; forms so small he estimated that 100 of these apparently tiny bits of life would not be as long as a grain of sand, or that they were more than 1,000 times smaller than the eye of a louse. These were his units of measurement in the study of microbial life.

Other equally minute organisms were observed in drops of stagnant water.

Some doubt exists whether Leeuwenhoek really described bacteria in this letter, but there is no doubt that he had observed bacteria when in a letter of 1683 he not only accurately described but pictured (see Fig. 1-3) as well the three main morphological groups known today, the cocci, the rods, and the spiral forms. In this and in later letters he also reported that many of these forms were actively motile, that their numbers rapidly increased under favorable conditions, and that they were widely distributed

in nature and could be destroyed by heat (few motile organisms could be observed in scrapings from his teeth after partaking of very hot coffee while many were present at other times). Truly Leeuwenhoek can be considered as the "father of bacteriology" if not "father of microbiology" itself.

**Bacteria and the Theory of Spontaneous Generation.** Early interest in bacteria centered to a considerable extent around arguments concerning the possibility of the spontaneous origin of life. To understand these arguments better, it is desirable to develop a general background of so-called "evidence" in support of such a hypothesis.

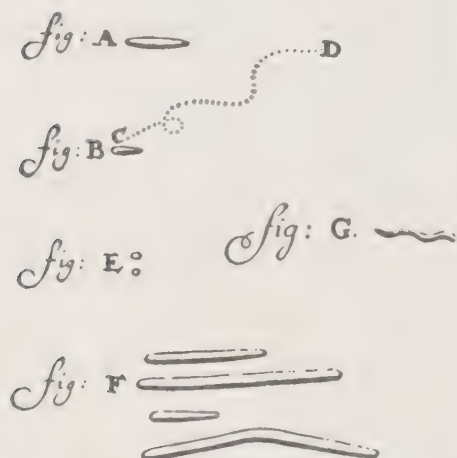


FIG. 1-3 Leeuwenhoek's drawings of bacteria, 1683. (From C. Dobell, "Antony van Leeuwenhoek and His 'Little Animals,'" John Bale, Sons and Danielsson, Ltd., London, 1932.)

Hundreds of years ago everyday experience gave "irrefutable evidence" that the sun moved around the earth. The casual observer saw that the sun arose in the morning, moved through the sky, and that night fell when the sun passed out of sight and was replaced by day again when the sun had supposedly completed its movement around the earth. "Evidence" of a similarly faulty nature pointed to the conclusion that under favorable conditions living things originate from lifeless matter. One day a plant, an animal, or an insect appeared where there was no evidence of its presence the day before. This was a "fact" so obvious that there was no need for a detailed study of the phenomenon. As early as 600 B.C. philosophers of the Ionian school postulated that living things could originate from amorphous slime under the influence of heat, sun, and air. Other Greek philosophers held similar views, but it should be borne in mind that their concept of spontaneous generation fitted in with their belief in the perpetuity of life. Some of the early philosophers simply conceived the entire universe to be living.

The views developed by Aristotle (384-322 B.C.) influenced scientific thought for many centuries. He postulated that living things are produced by the union of some passive principle, "matter," and an active principle, "form," this form being the soul of living things. Matter or substance by itself is devoid of life but is vivified by the energy of the soul. The soul is present in varying amounts in the elements—earth, water, air, and fire (heat)—of which living things are made, and their relative amounts control the nature of the life endowed by the soul. Thus, the earth endows plants, water aquatic animals, and the air terrestrial forms. The form of living things originating from their like depends upon "animal heat," but the form of those arising spontaneously from matter depends upon the sun's heat. Slime and decaying matter might possess some soul but do not of themselves produce living organisms, the fructifying influence of rain, air, and heat being required for the spontaneous generation of life.

Aristotle's teachings profoundly influenced the fathers of the Christian church and led to the acceptance of the theory of spontaneous generation by the church. To disbelieve then was to doubt the authority of the church. Basilus (315-379) taught that the earth originally produced plants and animals by the command of God and that this ability has been retained in full force; hence plants or animals may originate spontaneously from the earth. Saint Augustine (354-430) reasoned that God usually makes wine from water and earth by way of grapes but, as was done by Jesus on occasion, can dispense with the grapes and make wine directly from water. In like manner in the case of living creatures He can cause them to be born either from seed or from matter containing invisible seed. Thus the will of God may interfere with the normal sequence of events and be manifest in the spontaneous generation of life.

The medieval scholars corroborated the "facts" described by Aristotle of the origin of living creatures from decaying matter and supplemented these with fantastic observations or experiments of their own. In the seventeenth century observation of natural phenomena became more exact, but the concept of spontaneous generation was still in favor. For example, the famous alchemist and physician van Helmont left the following recipe for the spontaneous production of mice: "Place a dirty shirt in a vessel containing wheat and after twenty-one days' storage in a dark place, to allow fermentation to be completed, the vapors of the seeds and the germinating principle in human sweat contained in the dirty shirt will generate live mice." Strange to say these mice appeared to be replicas of natural mice arising from their parents!

Belief in the possibility of the spontaneous origin of the larger forms which bear their young alive began to weaken about this time, but faith in the spontaneous origin of the wee forms of life continued. Decaying



meat could, together with air and warmth, give rise to small white worms. The famous Italian physician, Redi, in 1668 exploded this idea when he demonstrated that these worms were nothing but fly larvae. By placing a screen of muslin between the meat and flies in the environment, he could show that although air did circulate freely and that the meat decomposed, yet no worms appeared. Worms developed in the meat when the flies were allowed to deposit their eggs thereon. He concluded that decaying matter merely offers a nest for the eggs and a suitable medium for their development but that the deposition of the eggs is a necessary preliminary event. Redi was unable to prove that still smaller forms of life could not originate spontaneously.

About this same time Leeuwenhoek was describing the world of living creatures invisible to the naked eye. He found that it was only necessary to place readily decomposable substances in a warm place for them soon to be teeming with wee beasties. If not a critical observer, one could see before one's eyes living matter originating in the decoctions. It is to Leeuwenhoek's credit that he did not entertain this view but supposed that they arose from animals already present in the material, in the container, or in the air in contact with the material. Leeuwenhoek was more interested in observing than in experimenting with such material, and it is to his follower, Joblot, that we owe the observation that such infusions teeming with microorganisms would no longer give rise to additional ones if boiled for 15 min. and stored in a closed container. When the cover of the container was removed, the infusion on standing once again quickly swarmed with microorganisms.

The French biologist Buffon and the Welsh priest Needham maintained that life can originate spontaneously in flasks of mutton gravy or other infusions which had been boiled and stoppered while still hot. An Italian, Lazzaro Spallanzani, in 1765 criticized these experiments, maintaining that Needham's vessels and their contents were not heated long enough and that they were not hermetically sealed by the corks employed for the purpose. He believed that air mechanically carried the germs of microorganisms and reported that when the soups were sufficiently heated and the flasks hermetically sealed, living forms did not appear.

Needham did not believe that he heated his liquids too little but rather that Spallanzani heated his too vigorously and thus destroyed a special creative force conceived to exist in the infusions. Needham contended that a special vital force exists in every organic molecule and supported Buffon's concept that living matter is composed of vitalized organic molecules which do not change but instead unite with each other in kaleidoscopic combinations to give rise to new forms of life. When a dead organism undergoes decomposition, its individual existence comes to an



FIG. 1-4. Portrait of Lazzaro Spallanzani.



FIG. 1-5. Theodor Schwann, father of the cellular theory.

end, but the molecules of which it was composed may recombine to form new living organisms.

Could it be that once a sterile vial of Spallanzani's soup was opened, it soon teemed with life because a vital force entered from the air and the creation of life became possible? The French chemist Gay-Lussac demonstrated that oxygen was lacking in vessels sealed while hot after their contents had been boiled. He also devised experiments to show that oxygen was necessary for life, taking part in respiration as had previously been suggested by Priestley and by Lavoisier. Experiments were devised to refute the presence of a vital force in air. In 1836 Theodor Schwann, the founder of the cellular theory, passed air through a heated tube into bouillon which had been boiled and demonstrated that spontaneous generation did not occur in the soup exposed to air which had been heated. Schulze in the same year conducted similar experiments but passed the air through strong acid rather than through a hot tube and obtained similar results. Proponents of the idea of a vital force in air claimed that heating or strong acids could destroy this force and hence spontaneous generation could not occur in the broths. In 1853 Schroeder and von Dusch simplified the experiment by filtering the air through sterile cotton which retained any dirt and microorganisms present in the air and yet subjected it neither to harmful chemical agents nor to heat. This study led to the present-day use in culture tubes of cotton plugs to permit free access of air to the cultures while at the same time preventing or greatly retarding contamination of the culture by other organisms.



In spite of the fact that these experiments tended to disprove the possibility of spontaneous generation, yet for some inexplicable reason microbes did occasionally appear in large numbers in some of the culture vessels. We know today that these failures were due to improper sterilization, to faulty vessels or plugs, and at times to the presence of bacterial spores, some of which can germinate even after many hours' exposure to boiling water. Actually the high degree of resistance of bacterial spores



FIG. 1-6. Ferdinand Cohn, discoverer of the heat resistance of the bacterial endospore.



FIG. 1-7. Louis Pasteur, the founder of modern bacteriology. (Portrait by E. Montgomery, courtesy of Central Scientific Co.)

to boiling did not become evident until 1877 when Ferdinand Cohn observed that the hay bacillus (*Bacillus subtilis*) formed small refractile bodies, endospores, which were heat-resistant and which could germinate and give rise to a new vegetative cell.

In an extensive work of over seven hundred pages, Pouchet in 1859 tried to show that spontaneous generation does occur, and he attempted to develop further the vitalistic theory of autogeneration formerly advanced by Needham and Buffon. The French Academy of Sciences offered a prize for exact and convincing experiments which would solve the problem. The prize was awarded in 1862 to Pasteur, who in a series of brilliantly executed experiments demonstrated that life did not originate spontaneously in various infusions and solutions of organic matter properly sterilized and protected from outside contamination.

Pasteur demonstrated the widespread distribution of microorganisms

in the air and concluded that air-borne microorganisms are a chief source of microbial contamination of fruit juices, broths, meat, vegetables, and various other foodstuffs. One of his experiments consisted in sterilizing broths in flasks with necks pulled out in the form of a letter S in a horizontal position. In these goosenecked flasks there was direct contact of the culture fluids with the outside air, but dust and germs in the air settled out in the bent portions of the neck and therefore the fluids were protected against contamination and remained sterile. A modern version of the Pasteur goosenecked flask is shown in Fig. 1-8. This flask and its content of nutrient broth were sterilized in 1942 and have stood on an open shelf in the laboratory of the author since that date with no evidence of bacterial or other microbial growth.

Since the air in the goosenecked flasks was not heated, filtered, or subjected to chemical treatment, there was no chance for a vital force to be altered or destroyed. Furthermore the broths were capable of supporting heavy growths of microorganisms, and it was only necessary to run some of the broth from the flask onto the dust in the outlet tube and then back into the flask again and in a matter of hours the broth

would be teeming with microscopic life. These experiments, together with semiquantitative ones showing the prevalence of microbes in the air over dusty areas and their scarcity in high altitudes, finally refuted the theory of spontaneous generation. The studies of Cohn on bacterial spores and of Tyndall on the absence of microorganisms in optically void air contributed to the final proof that organisms do not originate spontaneously in ordinary laboratory media.

From our present point of view this long controversy is hard to understand. But the belief in spontaneous generation is not entirely dead, and from time to time new "evidence" appears. After Pasteur's time the theory of spontaneous generation was resurrected for the origin of filtrable viruses, submicroscopic agents responsible for various infectious diseases of plants and animals. All evidence points to the improbability of life originating spontaneously from molecules chaotically dispersed in the ordinary broths of the bacteriologist. Yet how did life originate? Is

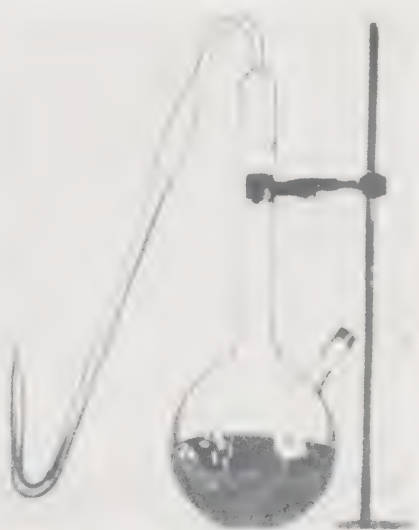


FIG. 1-8. Photograph of a modern form of the Pasteur goosenecked flask exposed to the air in the author's laboratory for several years without contamination.

spontaneous generation impossible? All we can say about the latter question is that no one has conclusively demonstrated that it can occur; our evidence is negative in that spontaneous generation has never been demonstrated conclusively; perhaps if the right conditions were provided, living things would appear.

The controversy over spontaneous generation stimulated interest in the study of bacteria and other microbial forms, and in these studies one may trace the development of modern methods for the observation and cultivation of the various organisms. But the interest went further than mere observation and cultivation of these forms, and students began to delve deeper into the mysteries of microbial life.

**Fermentation.** What are bacteria and what do they do? This question was a perplexing one for many years, and while we now know that they are to a great extent responsible for decay, fermentations, disease, maintenance of soil fertility, and numerous other activities in nature, it was only a little over one hundred years (1836) ago that Schwann, Cagniard-Latour, and Kützing independently proposed that fermentation is brought about by the activities of yeast cells. Before this time it was believed that fermentation was a natural decomposition of sugar in fruit juices and that fermentation may give rise to, rather than be caused by, microorganisms. The famous German chemist Liebig maintained that fermentation is a spontaneous process induced by unstable molecules in the fluid and that microorganisms may appear in the fermenting liquid since they are simply bits of loosely organized organic matter. Schwann's concept of fermentation gave rise to a bitter dispute between his followers, who supported the biological nature of fermentations, and those of the chemical school headed by Liebig. The concept that microbes are the causative agents of fermentations became more firmly entrenched as a result of Pasteur's studies on the "diseases" of wine and beer.

In 1848 Pasteur discovered that tartaric acid exists in two crystalline forms, one being the mirror image of the other. He could pick out the two crystalline types from a mixture, and he observed that when these forms were dissolved separately, one solution rotated the plane of polarized light to the left, the other to the right, while a mixture of the two in equal amounts had no apparent influence on polarized light. Ten years later he made the important observation that a microorganism (probably a *penicillium* mold) growing in a solution of ammonium tartrate utilized the dextrorotatory form while it did not attack the crystalline form (levorotatory) which in solution caused the plane of polarized light to be bent to the left. Inasmuch as these two crystalline materials were chemically the same and apparently differed only in crystalline structure,



Pasteur came to a realization of the remarkable specificity exhibited by microorganisms in their attack on organic matter.

Actually Pasteur developed an interest in fermentation at an earlier date, and his first paper on the production of lactic acid appeared in 1857. He noticed that a grayish material which appeared in solutions of sugar undergoing lactic acid fermentation<sup>1</sup> was composed of minute globules or very short rods and that the fermentation could be induced by transferring some of this material to fresh solutions of sugar. The process could be repeated in series, and Pasteur concluded that this "new yeast" was an organized living cell and that its action on sugar was essential for its development. Fermentation of sugar did not occur when the solutions were heated and the ingress of contaminated air prevented, nor did lactic acid-producing organisms develop.

In 1861 he turned his attention to the butyric acid fermentation and noted that this occurred in the presence of cylindrical rods. These bacteria were motile in the central portion of a drop of the fermenting liquid but ceased to exhibit motility at the edge of the drop in contact with air. To an inquiring mind this raises the question—is air inimical to the bacteria?<sup>2</sup> Pasteur was able to demonstrate that oxygen of the air was the agent inhibiting the activity of this organism. He discovered other organisms that could live without oxygen and in 1863 proposed that the terms *aerobic* and *anaerobic* be employed to designate cells which require air and those which do not, respectively. In 1866 he published his famous work "Etudes sur le vin," in which he showed that the spoilage of wine was due to the activity of microorganisms that elicited undesirable fermentations with the production of unpleasant odors or tastes in the wine. Pasteur was able to show that the undesired fermentations, or "diseases," of wine could be prevented by heating the wine at 55 to 60°C. after it was bottled, a process now known as pasteurization. These studies on diseases of wine led to the next logical step, the demonstration that diseases of living organisms can also be caused by undesirable microbial forms of life. This will be considered in a later chapter.

The demonstrations that microorganisms are the cause of fermentations and of infectious diseases—the latter idea strongly supported by the studies of Koch—and the arguments which Pasteur could so ably advance in support of his concepts firmly established the theory that living organized cells are responsible for a variety of both desirable and undesirable

<sup>1</sup> This terminology implies that the substance for which the fermentation is named is either the main or the most characteristic product rather than being the substance fermented.

<sup>2</sup> Actually Pasteur believed that these organisms were small animals and named them *Vibrio butyricus*. Throughout his studies his main interest was in the process rather than in an exact description and classification of the causative agent.

changes in living and dead matter. The interest in bacteria engendered by the observations of Leeuwenhoek and other microscopists and by the controversies over spontaneous generation was greatly enhanced by the studies of Pasteur, Koch, and their followers. Many investigators became interested in studying the nature of bacteria and their activities. This led to the development of modern bacteriology. Before we consider the



FIG. 1-9. Robert Koch, the father of medical bacteriology.

major characteristics of bacteria and of bacterial action let us attempt to define the term bacteria.

**Definition of Bacteria.** It is difficult to define the bacteria accurately. Any definition if made too narrow would exclude many organisms commonly considered to be bacteria; if made too broad it would include microorganisms other than bacteria. First of all we might describe them in a general way and then proceed to a workable definition. The bacteria constitute a large group of typically unicellular microorganisms placed in the class Schizomycetes (skiz-o-mi-se-tez, fission fungi). They are widely distributed in nature, being found in air, water, soil, the bodies of animals and

plants, and in dead organic matter. They exhibit three characteristic shapes (see Fig. 1-10)—spheres, rods, and curved forms, commonly known as cocci, bacilli, and spirilla, respectively. A few are filamentous or mold-like in appearance. Some possess flagella, by means of which they move about in an aqueous environment; some others have an amoeboid-like type of locomotion. A definite nuclear body is rather difficult to demonstrate, but chromatinic structures can be demonstrated within the protoplasm. Bacteria multiply rapidly by simple binary fission, although other types of reproduction may be observed in some species. Since they lack true chlorophyll, most bacteria are saprophytic or parasitic as regards their nutritional characteristics, but a few are autotrophic in character, obtaining energy from the oxidation of inorganic substances or from light. Bacteria are of the highest importance in the economy of life, many species converting complex organic compounds into inorganic ones or altering inorganic compounds, making them available as food for plants. The primary role of the bacteria appears to be that of scavengers, but many are of more direct value



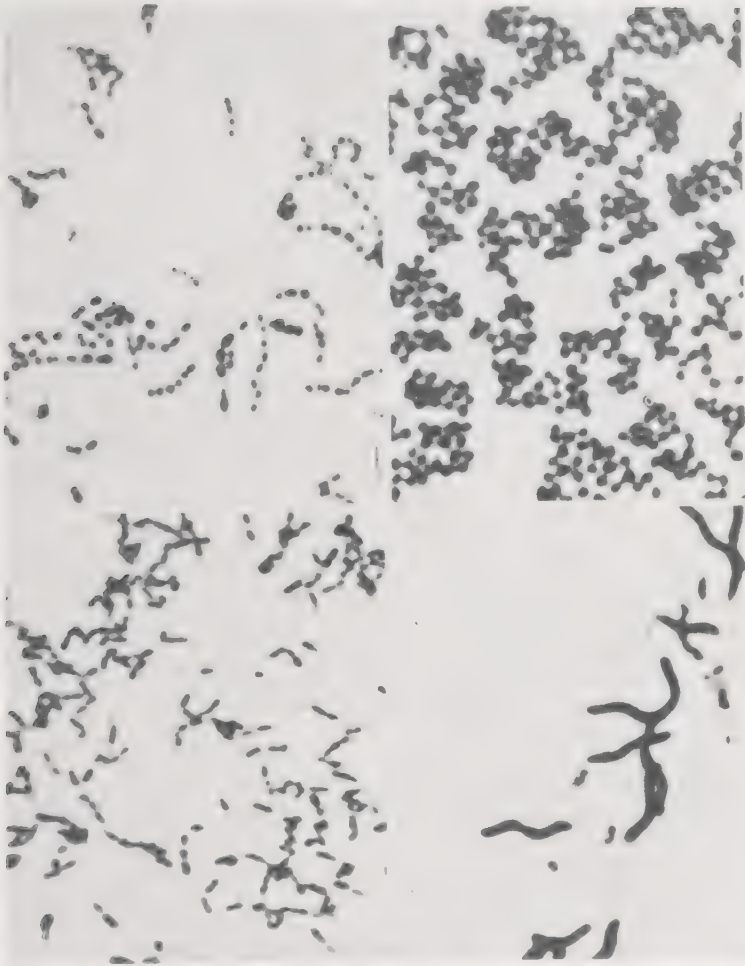


FIG. 1-10. Photomicrographs of cocci (upper left and right), rods (lower left), and spirilla (lower right).

to man, while a few are responsible for various infectious diseases of animals and plants.

If we take a somewhat middle position we can define bacteria as *extremely minute, rigid, essentially unicellular organisms; free of true chlorophyll and generally devoid of any photosynthetic pigments; most commonly multiplying asexually by simple transverse fission, the resulting cells being of equal or nearly equal size.* Let us analyze this definition in terms of its constituent parts.

To gain an idea of the minute size of bacteria, consider *Escherichia coli* (Fig. 1-11), a bacterium commonly studied in the laboratory and the predominant organism in the intestinal tract of man. A typical cell of this species is rod-shaped and is approximately  $2.0\ \mu$  in length by  $0.5\ \mu$  in breadth, a  $\mu$  (micron) being  $1/1,000$  mm. or approximately  $1/25,000$  in. Three hundred of these cells placed side by side would form a line

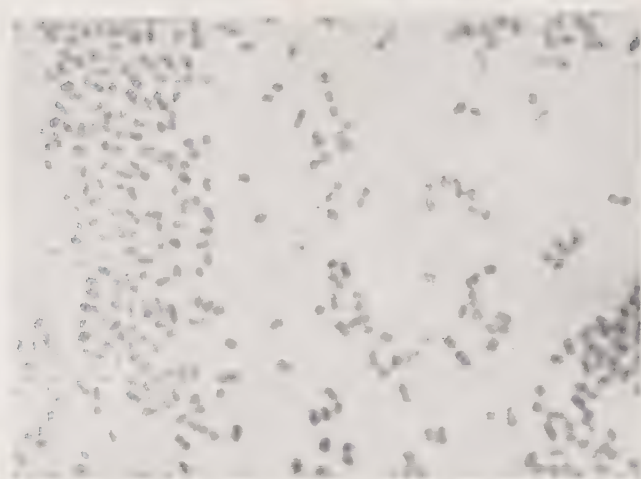


FIG. 1-11. Photomicrograph of *Escherichia coli*, approximately 1,000 $\times$ .

about the width of the field of view in a student's microscope equipped with an ordinary oil immersion lens and 10 $\times$  eyepiece. Or it would require about 50,000 of these microorganisms placed side by side to form a line 1 in. long, while 625,000,000 would cover an area of only 1 sq. in. A cubic inch could contain 31,250,000,000,000 bacteria of the size of *E. coli*. *Hemophilus influenzae* is approximately 0.2 by 0.5  $\mu$  in size, and therefore 125,000 cells side by side would form a line 1 in. long. To go on would soon lead us into figures of astronomical size! These minute organisms are generally observed with the aid of a microscope yielding an image nearly 1,000 times the linear size of the cell. Even then a single cell of *E. coli* appears to be but a speck, yet the average man magnified to the same extent would be about 1,500 ft. across his shoulders and 6,000 ft. tall.

How are such minute forms of life so able at times to disrupt the activities of man completely? Enormous numbers of bacteria per unit volume of their environment answer this question in part, but the ratio of surface area to mass appears to be of even more fundamental importance, since relatively enormous surface areas are involved. A man of average size weighs about 70 kg. and has a total surface area in the neighborhood of 1.5 sq. m. The ratio surface area weight in the case of man would be approximately 0.02 (1:50), while an organism such as *E. coli* with a surface of 0.000,004,5 sq. mm. and a mass of 0.000,000,000,5 mg. would have a surface area weight ratio of 9,000 (9,000:1). Since the metabolic activities of cells appear to be controlled to a great extent by this ratio, we can readily see why bacteria are able at times to consume per hour an amount of foodstuff equal to or even greater than their own weight, a feat that the greatest glutton could never approach. When we consider that there may be 1 billion bacteria in a milliliter of a culture and that they

could possess a total surface area of 1,000 sq. cm., we can readily see that while the change produced by one cell might be insignificant, yet the total change produced by such a number of cells with such a large total surface area could be of considerable magnitude. Bacteria are approaching the size of dispersed particles in the colloidal state, and since the properties of colloidal systems are to a great extent controlled by surface forces, we can see why the relatively great surface area of bacteria as compared with their mass assumes such importance, influencing not only metabolic activities but also other properties of cellular activity and behavior.

With organisms so minute as the bacteria it is extremely difficult to determine the internal structure of the cells. Each species, under a given set of conditions, tends to maintain a rather definite size and shape. This led observers to postulate that bacteria possess a definite limiting membrane or cell wall which imparts rigidity to the cell. If the bacterial cell in motion strikes a solid body, it may bend to some extent, i.e., it possesses a certain elasticity, but it tends to revert to its original form. Recent studies with plasmolyzed cells, with cells stained by special techniques, with the electron microscope, or with the aid of microdissection point to the existence of a definite, although very thin, cell wall. The cell wall imparts rigidity to most species of bacteria and is one characteristic of the bacteria which is more closely related to the vegetable than to the animal kingdom.

We have defined bacteria as essentially unicellular organisms, and this appears to be true as far as function is concerned. In many species, particularly when the cells are in what is known as the smooth form, cell division occurs by constriction of the cell wall, giving rise to single cells. When the cells are in the rough form, cell division occurs by the formation of cross walls, which subsequently split. Cell division may proceed at a slower rate than the deposition of cell walls, and this gives rise to a multicellular state. The multicellular condition is difficult to recognize in ordinary stained preparations but is readily evident when cell-wall stains are employed (see Fig. 1-12). Such a multicellular state, sometimes containing as many as twenty cells separated by septa and cross walls, has been observed in many species. In ordinary preparations, however, most bacteria appear to be in a unicellular state. Some organisms included with the bacteria do give rise to filamentous, branching forms that are truly multicellular.

The bacteria are classified as Schizomycetes in phylum I, the Thallophyta, of the plant kingdom. This signifies that they are fungi, and fungi are devoid of the chlorophyll of green plants. Certain bacteria, for example, the purple and green sulfur bacteria, do possess bacterial chlorophylls, photosynthetic pigments similar to the chlorophyll of green plants. The pigments allow these species to carry out types of photo-



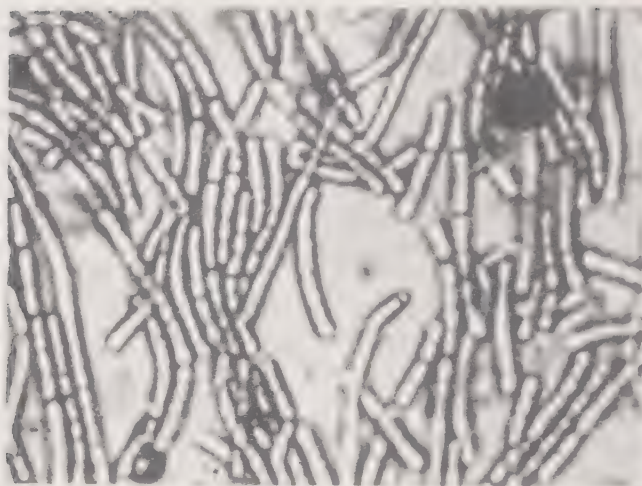


FIG. 1-12. Cell wall stain of *Bacillus megaterium*.

synthesis similar to, but not identical with, green-plant photosynthesis. However, the synthetic processes of the majority of bacteria are independent of light and in this respect are more closely related to the type of metabolism characteristic of the animal kingdom.

We have considered that bacteria commonly multiply by simple transverse or binary fission. Certain species of bacteria may reproduce not only by binary fission but also at times by other types of reproductive processes, particularly by formation of filamentous growths which later fragment by branching or by budding. Reproduction by "gonidia," sometimes of filtrable dimensions, has been reported in some species. Some of these methods may be sexual in character. Numerous observations, generally unconfirmed, of true sexual reproductive processes amongst the bacteria have been reported. The most reliable evidence for sexual reproduction comes from studies on crossing over between biochemical mutants of a species (see Chap. 14). Division of bacteria, however, most commonly occurs asexually and by simple transverse fission.

Consideration of cell division raises the questions: Do bacteria possess a nucleus, and does it divide during multiplication of a species? This has been a much-debated problem in the history of bacteriology. Numerous claims of the demonstration of a nucleus have been disproved, but new claims have constantly appeared. Many biologists, reasoning a priori from the structure of other cells, insisted that bacteria must possess a nuclear apparatus, but in cells so minute it is extremely difficult to demonstrate satisfactorily the presence of a true nucleus. Other workers claimed that bacteria possess a rather vaguely differentiated central body analogous to the central body, or rudimentary nuclear apparatus (in-

ipient nucleust), of the blue-green algae. Still others claimed, on the basis of staining properties, that the bacterial cell as a whole is primarily a nucleus, either as such or more generally as a diffuse nucleus. Numerous recent observations (see Fig. 1-13 and Chap. 3) with improved staining techniques, with the phase microscope, and with the electron microscope point to the existence of a nuclear apparatus in bacteria which under certain conditions can be demonstrated as a discrete nucleus. A few workers report that nuclear division occurs as in higher forms, mitotic

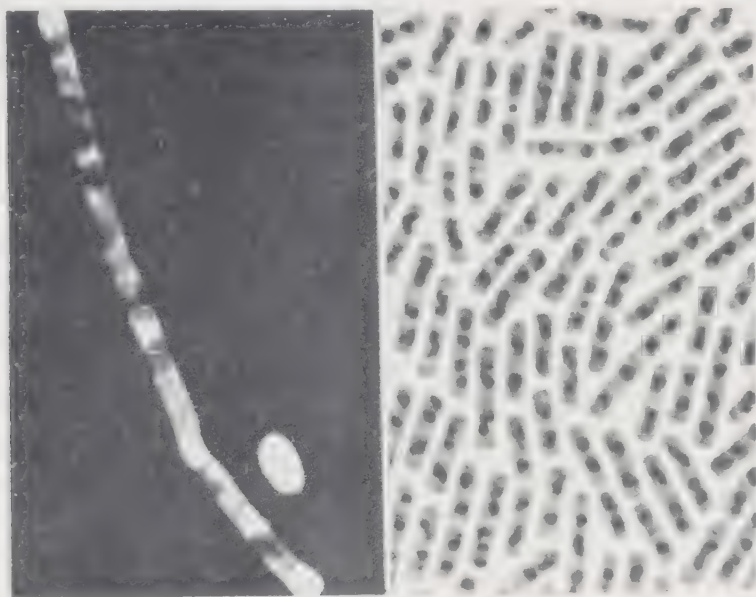


FIG. 1-13. Photomicrographs of the nuclear structures of a variant of *Bacillus anthracis* under the phase microscope and in a stained preparation.

figures and so on being reported. This point, however, is still open to debate.

**General Methods for the Study of Bacteria.** We have described and defined bacteria as extremely minute, rigid, essentially unicellular organisms; free of true chlorophyll and generally devoid of any photosynthetic pigments; most commonly multiplying by simple transverse fission, the resulting cells being of equal or nearly equal size. Such a definition has its merits as well as its faults, but will serve as a starting point for further consideration of the bacteria. We shall investigate, as we go along, the types of organisms which are roughly grouped together as bacteria and attempt to picture what they are and what they do—together, as far as possible, with a consideration of the how and why of microbial behavior.

First of all let us consider how we proceed in the general study of bacteria. One of the first observations we make is of the type of growth



exhibited by the species under consideration in liquid and on solid media. Some bacteria grow only at the surface of nutrient broth, forming a film or pellicle; others grow diffusely throughout the fluid, while many settle out of suspension readily, sometimes giving rise to sticky masses of cells. If individual viable cells are well separated on the surface of nutrient agar they give rise to distinct masses of growth known as colonies (see Chap. 11). The size, shape, color, and appearance of the colonies under the microscope may give some clue as to the identity of the organisms. But before final species identification can be established, it is frequently necessary to determine some of the physiological activities (type of material used as food or products produced therefrom) and even proteinaceous components (antigenic structure, see Chap. 22) of the cells. Before we proceed to considerations of these various procedures we should define certain terms and consider the more general methods employed in the study of bacteria in the laboratory.

One of the first observations of bacteria made by the beginning student in the laboratory is of the appearance of the cells under the microscope, both in their staining characteristics and general morphology. In the latter, one looks (Fig. 1-14) to see if the cells are spherical, rod-shaped, or curved. In addition, typical arrangements of the cells can be noted with many species, primarily as a result of their characteristic pattern of division. We have seen that division of bacteria appears to occur in either or both of two ways: the cell may constrict in the middle, thus

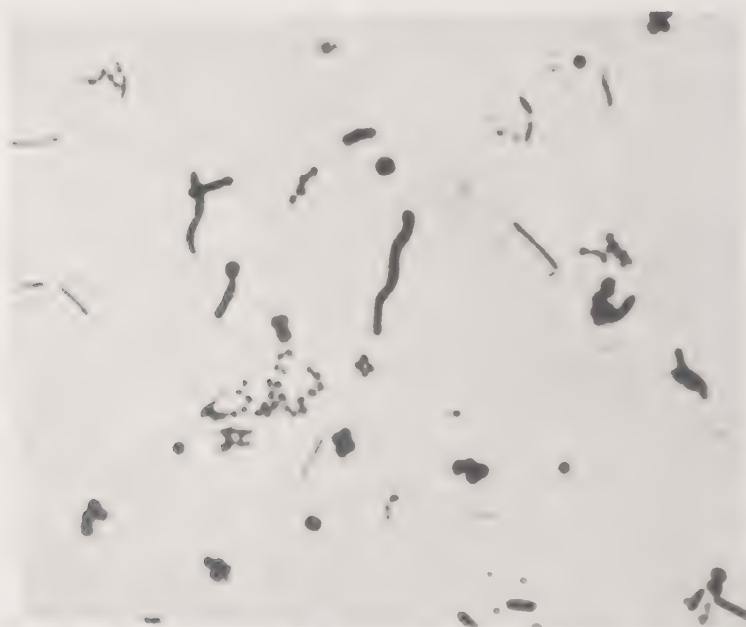


FIG. 1-14. Photomicrograph of a mixed smear of rods, cocci, and spirilla.

punching itself into two parts, or a cell wall is deposited transversely across the cell and this cell wall ultimately splits lengthwise. Once the cell wall has been formed the two cells may separate completely, or, particularly if the second type of division occurs, the cells may remain more or less firmly attached to each other. In the latter case the cells, particularly of the spherical bacteria, will show definite, characteristic groupings on microscopic examination.

Division of the spherical bacteria, while theoretically possible in any plane through the center of the cell, generally occurs in a definite plane or planes characteristic of a given species. When the cells remain attached after one cell division but separate before the next division is completed, the majority of the cells will be observed to be grouped in pairs. Cells characteristically showing this arrangement are known as *diplococci*. If, on the other hand, the cells tend to remain attached to each other through several divisions, they will be arranged as chains of cocci. When the majority of the cells are arranged in chains consisting of four or more cocci, they are spoken of as *streptococci*. Such cocci may appear to be somewhat elongated.

In other species of cocci, popularly known as *tetrads*, cell division occurs in two planes at right angles to each other forming a characteristic grouping of four cells arranged in a square. In other species division can occur in three planes at right angles to each other, giving rise to cubical packets of eight cells, the *sarcinae*. Finally cell division may occur at any angle through the center of the cocci giving rise to conglomerates of cells frequently appearing as grape-like clusters, cells showing this arrangement being commonly known as *staphylococci*.

Amongst the rod-shaped bacteria we find a few species characteristically arranged in pairs, the *diplobacilli*, or in chains, the *streptobacilli*, but the majority of species occur as free, independent cells. Observation of the arrangement of cells in stained smears is of considerable value in the identification and classification of the cocci; it is of little value with the rod-shaped bacteria and generally of no value with the curved forms which appear to separate immediately after division. In ordinary stained preparations of some species, clear areas will be evident in many of the cells. These generally represent endospores and should be looked for at the time one observes the general morphological appearance of the cells. In some cells these areas may represent deposits of lipids and it may be necessary to use specific fat or spore stains for identification.

The curved forms of bacteria may be roughly classified into two general morphological groups, the *vibrios* and the *spirilla*. The *vibrios* are but slightly curved and usually show only a single turn, somewhat resembling a comma in appearance. The *spirilla* are more markedly twisted than the *vibrios*, forming at least S-shaped cells; many species exhibit an even

more twisted structure. Some of the spirilla resemble the Spirochaetales, forms intermediate between the true bacteria and the Protozoa, which possess a definitely spiral or coiled structure and are flexible rather than rigid cells.

In the next chapter methods for the observation of bacteria under the microscope will be discussed. Since we have defined and described bacteria in general terms, we should also define certain other terms and consider the more general cultural methods commonly employed in the study

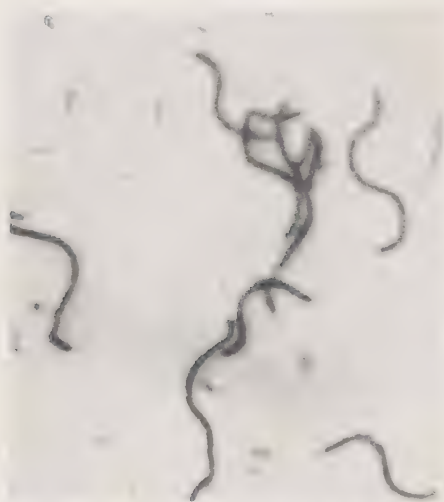


FIG. 1-15. Photomicrograph of *Rhodospirillum rubrum*, a facultatively photosynthetic spirillum.

of bacteria in the laboratory. Observation of bacteria under the microscope is of value in the study of these minute forms of life, but much of our information comes from studies of their behavior in cultures. A *culture* may be defined as the growth of microorganisms in or upon a suitable nutrient medium. The majority of bacteria can be cultivated in nutrient broth, which is primarily an aqueous extract of meat frequently enriched with peptones (products of partial hydrolysis of proteins), with extracts of yeast or other cells, or with blood. These substances supply certain nutrients (foodstuffs) and other complex substances (vitamins) needed for the

growth of those organisms with fastidious or complex dietary requirements. On the other hand, a limited number of bacteria will grow in a purely inorganic culture medium.

A *pure culture* consists of only one species of microorganism growing in or on the culture medium. Pure cultures are seldom encountered in nature, and the bacteriologist must separate the mixed population into its separate components or isolate the organism desired for study from the other organisms associated with it. This is generally done by streaking a bit of the material, held on a previously sterilized inoculating needle, over the surface of nutrient agar, giving rise to what is known as a *streak plate* (see Fig. 1-16). Nutrient agar consists essentially of nutrient broth to which sufficient agar has been added to produce a semi-solid material which resembles a rather stiff jelly. Instead of streaking the surface of the agar with bacteria, they may be mixed with molten nutrient agar (from here on these nutrient media will be referred to simply as agar or broth) and the mixture poured into a sterile dish where



FIG. 1-16 Streak and pour plates showing how bacteria can be separated from each other and give rise to separate colonies.



it is allowed to solidify (*pour plate*). The purpose of pour or streak plates is to separate the various organisms mechanically and thus to permit, as far as the limitations of the methods allow, each cell to grow by itself. On standing, many of the organisms will multiply and give rise to characteristic growth, or *colonies*. When these colonies are well separated, it is generally found that each one is composed of an individual species of bacteria. Representative colonies may be picked (fished) from the agar by means of a sterilized needle and again streaked on agar to determine if only one type of colony or organism was present in the parent colony. Microscopic examination is also of aid at this point in the determination of the purity of a colony. Also microscopic methods have been developed for the isolation of single cells and their transfer to nutrient media for the establishment of *pure-line cultures*, cultures derived from a single cell.

Once an organism has been obtained in pure culture, it can be maintained as such either in broth or on agar slant cultures. *Slant cultures* are cultures in which the organisms are growing on the inclined surface of a solid medium such as agar, gelatin, coagulated blood, or slices of vegetables such as potatoes or carrots in a test tube. These slanted media are generally inoculated by streaking the surface of the medium in such a manner as to obtain heavy growth of the organism. The advantage of slant cultures is that a relatively large area of culture medium exposed to the air may be obtained in the small volume of space occupied by the test tube. Needless to say, all nutrient media must be free from living organisms before they are employed for the cultivation of pure cultures and must be handled in such a way as to prevent the entrance of organisms other than those under study. The cotton plug in test tubes and flasks of media serves as a mechanical barrier to prevent the entrance of organisms from the external environment and was developed, as was just considered, from studies on spontaneous generation.

A few microorganisms do not grow readily, if at all, when exposed to the air. These organisms may grow if the oxygen of air is removed from the culture vessel by mechanical or chemical means. One simple way of obtaining growth of organisms in the absence of air is to prepare *shake* or *stab* cultures. A stab culture is one in which the organisms have been inoculated in the depths of the solid medium by stabbing the medium to a depth of at least 2 in. with a needle bearing some of the desired organisms. A shake culture is one in which the organisms have been added to a test tube containing the molten medium and thoroughly mixed with it before it solidifies. Organisms (*aerobes*) which require oxygen will grow on or near the surface of stab or shake cultures; others (*anaerobes*) to which oxygen is harmful will grow only in the depths of the medium; still others (*facultative anaerobes*) will grow throughout the medium.



FIG. 1-17. Photographs of (A) stab, (B) slant, and (C, D) broth cultures of bacteria. Growth is profuse throughout C but occurs primarily as a film or pellicle on the surface of the broth in tube D.

Once an organism is obtained in pure culture and its morphology and staining properties are ascertained, the next step in its identification is a study of its physiological activities. This is made by adding a desired test chemical to the medium or fluid in which the cells are to be grown and determining whether or not and in what manner the substance is utilized by the cell. For example, one organism may utilize a sugar such as sucrose (cane sugar) as a source of energy, and under anaerobic conditions (absence of oxygen) ferment it with the production of acids which can be detected with the aid of a hydrogen-ion-concentration indicator. A second organism may ferment sucrose with the production of both acid and gas, the latter being detected by its displacement of broth in a small tube inverted in the larger culture tube or as bubbles produced in a stab or shake culture. A third organism may not be able to utilize the sugar as a fuel, and hence no acid or gas is produced. Thus we have a means (see Fig. 1-19) of identifying these three organisms which in colony form and structure and under the microscope may appear so alike as to render their identification impossible. The ability of organisms to utilize other sugars, alcohols, and amino acids, or their production of specific products therefrom, increases the possibility of our identifying these organisms by



FIG. 1-18. Schematic illustration of shake cultures of different species of bacteria. Organism *A* is an aerobe and grows only at the surface, *B* is a strict anaerobe growing only in the depths of the medium, and *C*, a facultative anaerobe, grows throughout the medium. The broken spaces in *B* and *C* indicate that gas is produced by these organisms under anaerobic conditions.

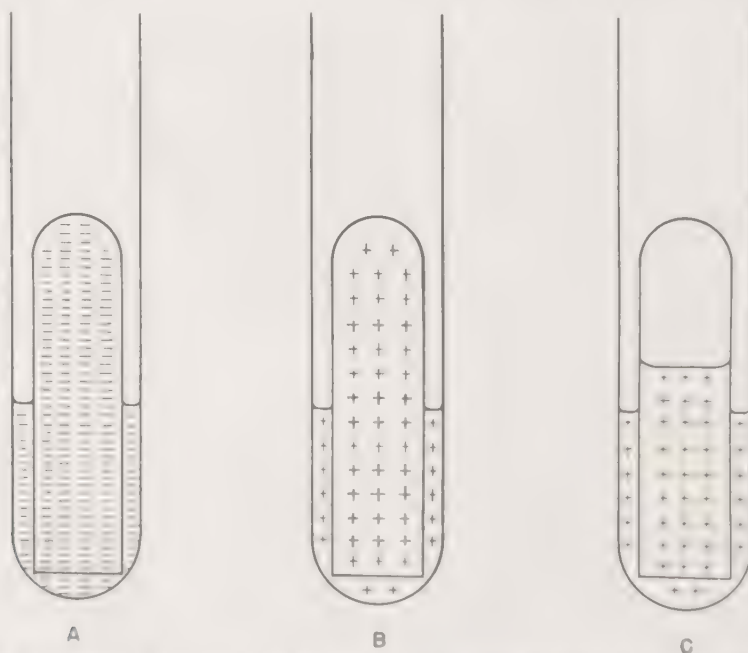


FIG. 1-19. Schematic illustration of fermentation characteristics of different species of bacteria. — represents growth without acid production, + with acid, and the clear space in one inverted tube represents gas formation.

the changes produced by pure cultures in nutrient culture media. Observations of the morphology of the cells, of their staining properties, of the colonies they form on agar, and of the physiological or biochemical behavior of pure cultures of bacteria are two important tools in the study, identification, and classification of these minute forms of life.

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## CHAPTER 2

### THE OBSERVATION OF BACTERIA

The first step in the development of the science of bacteriology in reality dates from the invention around 1590 of the compound microscope, the origin of which is generally attributed to the Janssens, Hans and Zacharias, of Middelburg, the Netherlands. At the time of Leeuwenhoek's discovery of bacteria the compound microscope was such a crude instrument that Leeuwenhoek employed simple microscopes in his studies. With increased understanding of the optical principles involved, together with mechanical improvements and different types of lenses, the microscope was steadily improved. Great progress was made during the last century with the development of the water-immersion lens by Amici in 1840 and his subsequent development of the oil-immersion lens in 1869; the substage condenser by Abbe in 1872 which provided better illumination by bringing the rays of light into focus in the object under examination; and the introduction by Abbe and Zeiss in 1886 of lenses that were fully color corrected (apochromatic objectives with compensating eyepieces).

#### MICROSCOPY

In our daily life we continually magnify objects without employing a lens. The letters on this page are illegible at a distance of 100 in., but they become easily readable when magnified 10 diameters by bringing the page 10 times closer. As you bring this book still closer, the letters are further magnified, but a limit is soon reached beyond which the letters become blurred. The eyes are unable to focus on an object which is held too close to them. When a simple microscope, a low-power convex lens, is placed at an appropriate distance between the eye and the object, the latter once again becomes clear and appears to be of greater size. The reading lens enables the eye to bring into focus the image of the object as formed by the lens.

The compound microscope differs from the simple system just described in that instead of making it possible to bring the object nearer the eye an enlarged image of the object as produced by the objective lens is brought nearer to the eye with the aid of the eyepiece lens. The eyepiece lens acts

as a simple microscope and thus makes it possible for the eye to focus at close distance upon the enlarged image of the object being examined.

The approximate magnifying power, expressed as increase in linear size, is determined by multiplying the magnifying power of the eyepiece

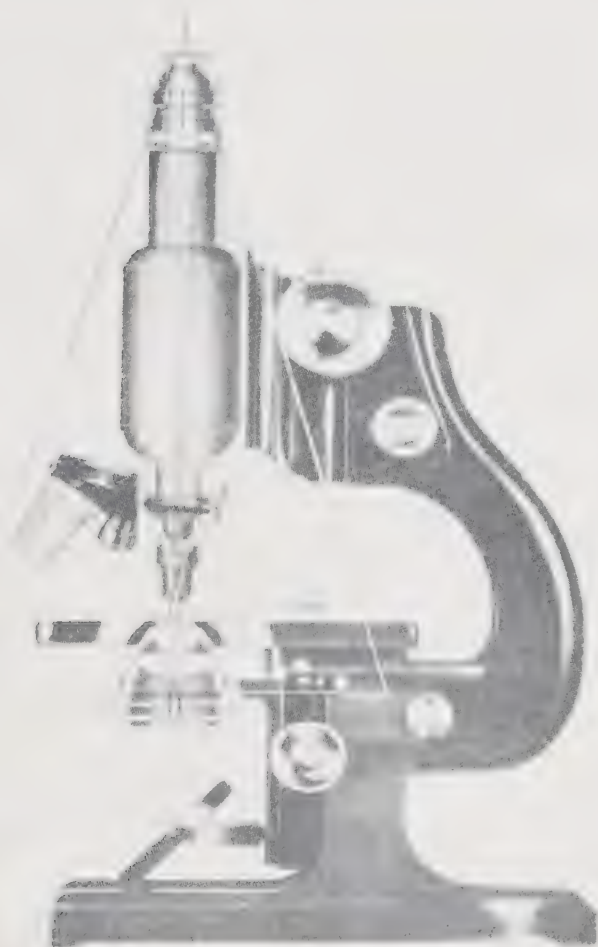


FIG. 2-1. The compound microscope. (Courtesy of the American Optical Co.)

(usually  $10\times$ ) by the magnifying power of the objective. Thus, when used with a  $10\times$  eyepiece, the low-power ( $10\times$ ), high dry ( $43\times$ ), and oil-immersion ( $97\times$ ) objectives give linear magnifications of 100, 430, and 970 diameters, respectively.

One would think that with nearly perfect lenses it would be possible to employ compound microscopes in series with each other and thus obtain still higher magnifications, which in turn would enable one to see still smaller objects. It is true that such instruments have been built, and

they do magnify several thousand times, but objects smaller than those visible in the best ordinary compound microscope cannot be seen. The ability to magnify does not by itself make the microscope an effective instrument. Something more is needed—resolution.

By resolving power is meant the ability to show, separately and distinctly, two points which are closely adjacent in the object. The resolving power of a microscope is determined by two factors, the wavelength of light and the numerical aperture of the lens. The wavelengths to which the eye is sensitive are fixed, and there are limits beyond which the numerical aperture of a lens cannot be increased. The numerical aperture is dependent upon the actual diameter of the objective lens in relation to its focal length and upon the light-bending power (refractive index) of the medium between the lens and the object under examination. Immersion oil has a higher index of refraction than air, and hence higher resolving power is obtained when oil is placed between the immersion lens and the object, but a limit of attainable resolving power is soon reached beyond which increased magnification does not enable one to see still smaller objects. We can picture the resolving power of a lens as being limited by the fact that the image of a point is not actually focused as a point but instead as a small disk. The smaller this disk, the more nearly it approximates a point, the greater is the resolving power of the lens. If the object is smaller than can be resolved by the lens, the disk images from points about the object overlap, and the object is obscured.

Resolving power of a lens may be expressed by the equation

$$\text{Resolving power} = \text{smallest visible structure} = \frac{\text{wavelength}}{\text{numerical aperture}}$$

When the microscope is fitted with a condenser with a numerical aperture equivalent to that of the objective, the equation becomes

$$\text{Resolving power} = \frac{\text{wavelength}}{2 \times \text{numerical aperture}}$$

On substitution in the above equation we find that in blue light of wavelength  $0.470 \mu$  the resolving powers of lenses ordinarily employed in bacteriological laboratories are as shown in Table 2-1 on page 29. When one employs an ordinary student microscope equipped with a good condenser and an ordinary oil-immersion lens, the smallest object which can clearly be seen must have a diameter of at least  $0.2 \mu$ .

For increased resolution it is necessary to use light of shorter wavelength, and this can be accomplished with quartz lenses which are transparent to ultraviolet light. However, the eye is not sensitive to the short ultraviolet and it is therefore necessary to photograph the image and to

TABLE 2-1

	Numerical aperture	Resolving power, $\mu$
Low-power lens.....	0.25	0.94
High-power lens.....	0.85	0.28
Oil-immersion lens.....	1.25	0.19
Research oil-immersion lens...	1.40	0.17

observe the photographic reproduction. Using light of  $0.275 \mu$  wavelength, the resolving power becomes approximately  $0.1 \mu$ .

**The Phase Microscope.** Contrast in the images of bacteria or other specimens can be enhanced over that noted in an ordinary microscope by use of the phase-contrast microscope. Light traversing two objects will emerge out of phase if one object is either thicker than or has a different refractive index from the other. When these rays are brought together out of phase, they interact to produce interference or darkening; when brought into phase, they reinforce each other and yield a brighter image. In the phase microscope a diffraction plate or coating is added within the objective and an annular diaphragm below the condenser of a light microscope. This optical arrangement is of such a nature that slight and otherwise invisible alterations of the light passing through the specimen are converted into images that can be seen. The annulus below the condenser controls the illumination on the diffraction plate where the light from the specimen is selectively modified to yield an image of greater visibility or contrast. On changing the nature of the annulus or of the diffraction plate, changes can be induced in the degree of contrast, or the contrast can be reversed, thus altering the appearance of the object under examination. Phase-contrast microscopy has definite limitations, but within these limits it is of value in enabling one to observe differences in structure of living cells that are not apparent in the ordinary light microscope. For example (Fig. 1-13), it is possible to follow changes in the appearance of nuclear structures during the growth of bacteria.

**The Electron Microscope.** In recent years it was observed that electrons can be deflected from their course by magnetic fields (see Fig. 2-2) in a manner analogous to the deflection of light by lenses. Since electrons moving at high velocity have a very short wavelength ( $5 \times 10^{-6} \mu$  at 50 kv. potential) the resolving power of the electron microscope is approximately 100,000 times that of a light microscope. Unfortunately the "objects" seen in photographs of electronic images are shadows analogous



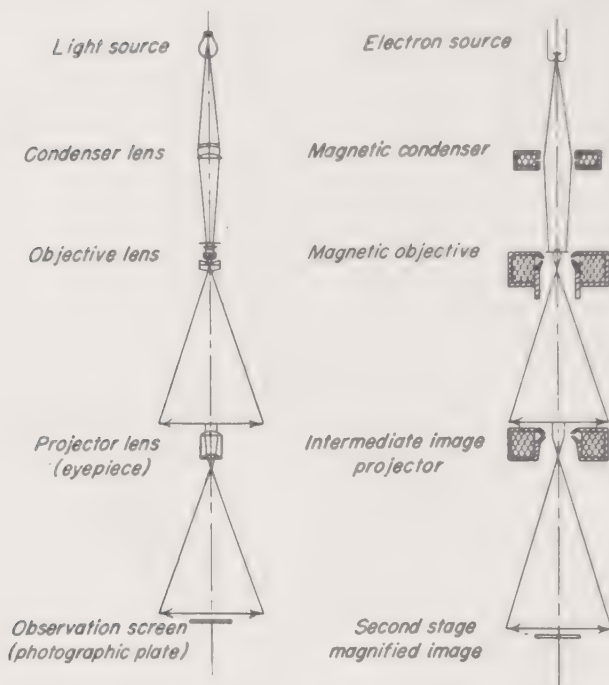


FIG. 2-2. A comparison of electron and optical microscopes. (Courtesy of the Radio Corporation of America and John Wiley & Sons, Inc., from V. K. Zworykin, G. A. Morton, E. G. Ramberg, J. Hillier, and A. W. Vance, "Electron Optics and the Electron Microscope," New York, 1945.)

to those on an X-ray plate and represent degrees of opacity to electrons by different parts of the object. Also the specimen under examination must be held in a high vacuum, and this may tend to distort bacteria or bacterial structures. In many instances, interpretations of the photographs (electron micrographs) are difficult to make, but notwithstanding all the difficulties encountered in electron microscopy the use of this tool has increased our knowledge of the structure of bacteria and viruses. Electron micrographs of bacteria are compared in Fig. 2-3 with ordinary light photomicrographs of bacteria. Electron microscopy has also been of considerable value in the determination of the size and shape of virus particles. A recent valuable development by Wyckoff and others consists in depositing a thin film of metal on one side of the particles so that they cast shadows. This technique makes the particles show up more prominently.

**The Dark-field Microscope.** When a beam of light shines through a darkened room, we see light reflected in all directions by dust and other particles suspended in the air. The suspended particles may be so small as to be invisible to the naked eye, and then we see only the *light reflected by the particles* and not the particles themselves. This optical effect, known as the *Tyndall phenomenon*, can be applied to microscopy and

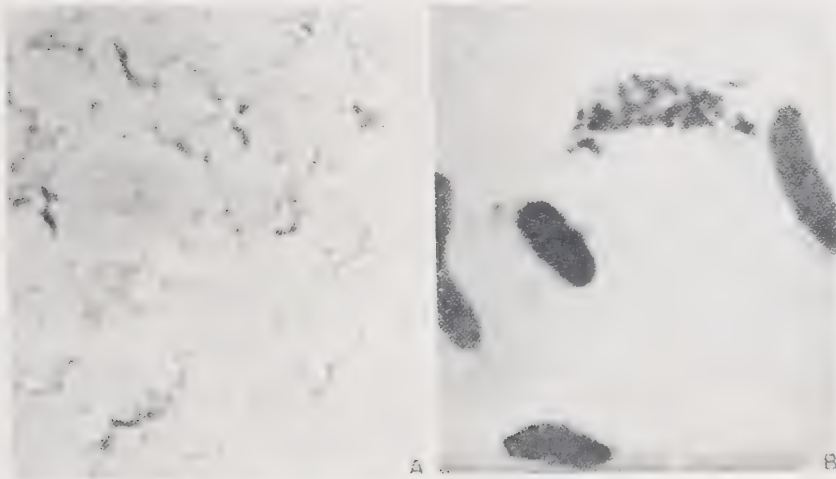


FIG. 2-3 Comparison of (A) an optical- and (B) an electron-microscope view of a *Pseudomonas*, magnification approximately  $2,000\times$  and  $10,000\times$ , respectively.

enables one to detect the presence of particles, even though their dimensions are less than the lower limits of resolution of the microscope. This is accomplished by providing a dark background through which a beam of light passes at a right angle to the optical axis of the microscope and serves as the basis for the ultramicroscope of the colloid chemist. A modification of this original type of ultramicroscope is commonly employed in the bacteriological laboratory.

Dark-field illumination is obtained when the usual condenser of the microscope is replaced with a special dark-field condenser. The path of the rays of light through this type of condenser (see Fig. 2-4) is such that the rays are brought into focus in the object but at such a divergent angle that none of them strikes the objective lens of the microscope. Only rays of light reflected by particles in the field of view are able to enter the objective.

When one looks through a microscope equipped with a dark-field condenser at a drop of water, the field of view appears dark. When bacteria or other objects that will reflect light are present in the drop, some of the reflected light will pass through the objective and form an image. One then sees the object as an apparently luminous body outlined against the dark background. Since the surface of the bacterial cell and structures within the cell reflect light to different extents, one may obtain some idea as to the structure of the cell. Also one can readily observe motion of the cells in the drop of suspension medium. Bodies below the limits of resolution of the microscope may reflect light and thus give rise to points of light which can be counted or observed to be in motion. Hence with the aid of a dark-field microscope it is possible to detect the presence of subvisible particles such as filtrable viruses, although in a heterogeneous system it is impossible to differentiate between viruses and other particles.

Dark-field microscopy is particularly valuable for the observation of the motility of bacteria and for the identification of *Treponema pallidum* in material from suspected syphilitic lesions.

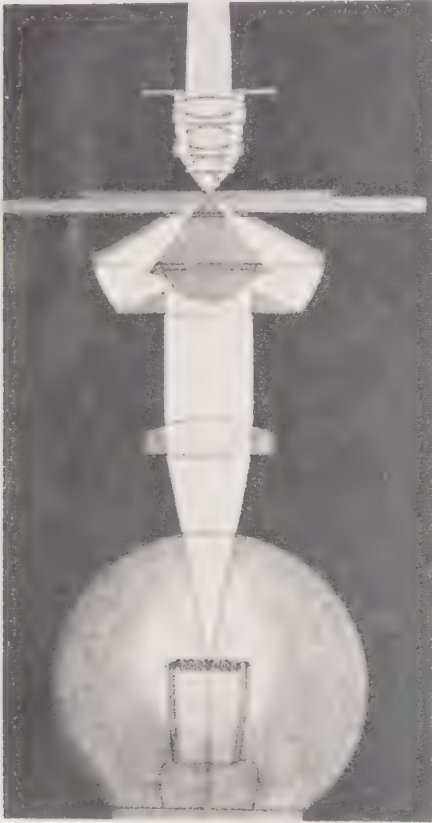


FIG. 2-4. Schematic drawing of dark-field illumination. (Courtesy of the American Optical Co.)



FIG. 2-5. Dark-field photomicrograph of bacteria and spirochetes in scrapings from around the teeth.

**Microscopic Examination of Bacteria.** It is necessary, because of the small size of bacteria, to use high-power objectives, particularly the oil-immersion lens, to obtain sufficient magnification and resolution for the examination of these and related microorganisms. The principle involved in the use of the oil-immersion lens is illustrated in Fig. 2-6. Microorganisms can be examined as masses in living colonies or cultures or as individuals in suspension in water or in dried stained preparations. The latter method is usually employed since the unstained cells, particularly bacterial, are generally transparent and the refractive index of protoplasm is so near that of water that it is difficult to observe such minute colorless cells. Staining is also useful in revealing flagella and capsules or structures such as granules and spores within the cell.

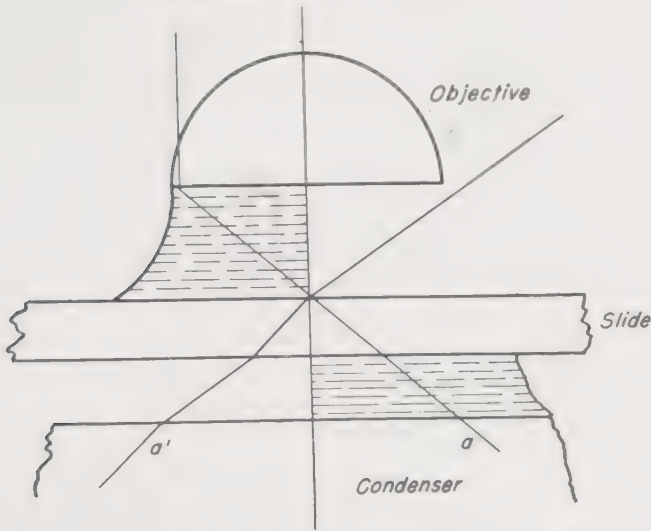


FIG. 2-6 Influence of immersion oil with oil-immersion lenses. Since the oil (—) has the same refractive index as glass, a light ray ( $a$ ) will not be bent on passing from the one medium into the other, whereas an equivalent ray ( $a'$ ) not passing through oil would be refracted in such a manner that it would not enter the objective. The oil, therefore, increases the amount of light which will pass through an object and into the objective. (Oil is employed between the slide and the condenser only for critical illumination.)

The general method for staining bacteria is to place a small portion of a culture in a drop of water on a microscope slide, mix thoroughly with an inoculating needle, and spread the drop in a uniform layer over the surface of the slide. When the bacteria are in a broth culture, a drop of the culture can be placed directly on the slide and spread out as above. The preparation is allowed to dry in the air, and the cells are then fixed to the slide by passing it, smear uppermost, through a bunsen burner flame three or four times. The fixed organisms are then stained by placing several drops of the dye solution on the smear and allowing the dye to act for a few seconds or minutes, the length of time being dependent upon the nature of the dye and of the bacteria. The excess dye on the slide is then washed off with water, the preparation dried by placing it between pieces of filter or blotting paper, and it is then ready for examination under the microscope. One observes the preparation with particular reference to the relative size and shape of the cells and their arrangement in the smear and for variations in these properties amongst the cells of a particular species. Under a given set of conditions the size and shape of the cells of a species may vary with the age of the culture and with the nature of the culture medium. Bear in mind the fact that in the laboratory the cells are growing in a constantly changing environment (changes produced by metabolic activities of the bacteria) and that variation is to be observed in all forms of life. It becomes particu-



larly evident with these small organisms which have generation times as short as 15 min.

Certain staining reactions are employed to bring out differences in the staining properties of specific structures within the cell or differences between different species. These will be discussed later, but one special staining technique may be mentioned at the present time. If one mixes some bacteria with a drop of Congo red, nigrosine solution, or India ink, spreads a drop of the suspension on a clean slide to form a thin film, and dries it, the bacteria may be seen as colorless bodies surrounded by a colored background. This is called



FIG. 2-7. Photomicrograph of bacteria in a negatively stained preparation.

*negative staining*, since the background and not the bacteria is colored. The cells appear to be larger than in ordinary stained preparations, since in the latter process there is a tendency for the cells to shrink in the fixing and staining procedures. On the other hand, the dye may pull away from the cells during the time that the negatively stained smear is drying and create the illusion of increased size. Negatively stained preparations are useful for certain types of work as they tend to be less tiring to the eyes, the bacteria stand-

ing out as bright spots in a dimly lighted field.

**Hanging-drop Preparations.** Unstained organisms can be examined under the microscope in a drop of water or broth, generally covered with a cover glass to form a thin film. Since the cells have a refractive index near that of water, it is necessary to adjust the illumination so as to obtain as great contrast as possible between the cells and the suspension medium. One may add a drop of a dye solution or of dilute iodine to the preparation to increase the contrast, and in some instances a so-called *vital stain* which will penetrate living cells may be employed.

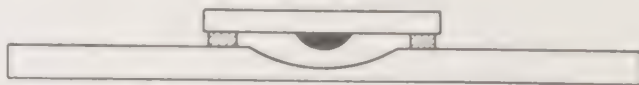


FIG. 2-8. Cross section of a hanging-drop preparation for the observation of bacteria in a fluid.

It is generally preferable to examine living bacteria in what is known as a *hanging drop*, in order to reduce movement caused by capillary forces or by evaporation from the film under the cover glass. For this prepara-

tion a glass slide with a concave depression is required. A thin film of petroleum jelly is applied around the edge of a cover slip, and a drop of the bacterial suspension is then placed in the center of the cover slip. The hollow-ground slide is then inverted over the cover glass in such a manner that the drop is centered over the depression in the slide. The slide is then gently pressed down on the cover glass to form a seal of petroleum jelly between it and the edges of the cover glass. The sealed preparation is rapidly inverted, and one then has a drop of the suspension hanging from the cover glass. Sealing the preparation greatly reduces evaporation from the drop and at the same time markedly reduces convection currents. The organisms are now ready for examination, preferably with the high-power lens, although the oil-immersion lens can be employed if reasonable care is exercised. It is generally desirable to reduce the illumination and to locate the drop with the low-power lens before shifting to the higher powers of the microscope.

**Motility.** Hanging-drop preparations are valuable for the determination of motility of bacteria. Amongst the bacteria, motility is for the most part due to the possession of whip-like organs of locomotion, *flagella*. Bacteria can be roughly classified into two groups, motile and nonmotile, on the basis of motility in hanging-drop preparations. Even nonmotile bacteria in a suspension are in constant motion, but one must learn to distinguish between this motion and true motility. Brown in 1828 observed that pollen grains in suspension in water were in constant motion, a motion which can best be described as a vibration or trembling around a point. This phenomenon, known as *Brownian movement*, is caused by the continuous bombardment of suspended particles by the molecules of the suspending medium, generally water or aqueous solutions. At any one time there may be by chance more or stronger hits on one side than on the other sides, and the particle will be displaced to a slight extent. At the next instant the bombardment may be of greater intensity on another surface of the particle, and the particle will be displaced in such a direction as to minimize the applied force. This displacement is generally of a low order of magnitude, and with unequal bombardment from different directions at different times, the particles will be in a constant state of motion around a point, although occasionally by chance a particle may be displaced through a considerable distance in the field. Brownian motion closely resembles that of a swarm of gnats "dancing" in the air. When bacteria possess flagella, they may exhibit true motility as well as Brownian movement. True motility can be distinguished from Brownian movement by the fact that a motile organism may be observed to move from one place to another in a more or less straight line and that this movement may carry the organism partly or entirely across the field of view. The velocity of motile bacteria can be quite high when com-

pared with their size, a typical organism moving at a rate equivalent to six times its own length per second, the corresponding velocity for a horse being 30 miles per hr. However, the actual rate of movement is generally only a few inches per hour, although motile bacteria may appear to move very rapidly under the high magnification employed in observing them. Not all motile bacteria exhibit true motility all of the time, and in fact one may have to examine a hanging-drop preparation for several minutes before true motility is observed. One reason for this becomes apparent when we consider that if we were observing from a distance a herd of animals, all might remain stationary for a period of time, a few might shift their position from time to time, or all might be in motion at any one time. One must remember that bacteria in suspension always exhibit Brownian movement and that it is necessary to distinguish between this movement and true motility. Also bear in mind that motility of bacteria is greatly reduced or lost with increasing age of a culture and that handling of the organisms may determine whether flagella remain attached to the cells. Loss of flagella means loss by the cell of its mode of locomotion.

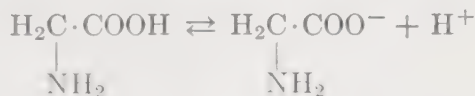
#### GENERAL STAINING PROPERTIES OF BACTERIA

Bacteria are for the most part colorless organisms and are difficult to observe in unstained preparations. Stains were first employed in 1858 by Gerlach to increase the contrast between cells and their constituent parts in slices of animal tissues. The staining technique was introduced into bacteriology by Weigert in 1875 when he demonstrated that bacteria can be more readily observed under the microscope after preliminary staining with methyl violet. New stains and staining techniques have been developed since Weigert's time, but before we consider the nature of the staining reactions, it is necessary to summarize the nature of dyes and of the proteins with which they may react in the cell.

**Proteins.** The chemical properties of proteins are in large part dependent on the properties of the amino acids of which they are composed. Proteins vary widely amongst themselves in the relative amounts of the 21 amino acids of which they are commonly constituted. The proteins are compounds of high molecular weight and are probably the most complex chemical entities known to man. They are the main organic constituent of protoplasm, and their complexity accounts in part for many of the remarkable properties of living matter. Proteins always contain carbon, hydrogen, nitrogen, and oxygen and usually small amounts of sulfur, iron, and phosphorus. In the cell, proteins may also be linked with other complex chemical substances such as carbohydrates, fats, and nucleic acids and with inorganic salts.

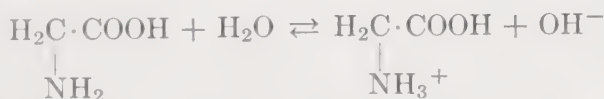


Amino acids are members of a group of substances known as *amphoteric compounds*, or *ampholytes*, which possess the property of ionizing either as an acid or as a base. The simplest amino acid present in proteins is glycine (aminoacetic acid). When it reacts as an acid, its behavior may be depicted as



glycine dissociating to form a negatively charged amino acid ion and a hydrogen ion. Amino acids react in this manner in the presence of a base such as sodium hydroxide and give rise to the formation of a sodium salt of the amino acid and water.

In an acidic solution, amino acids react like a base and dissociate to give a positively charged amino acid ion and a hydroxyl ion. Glycine behaves as a base similar to ammonia, which unites with water and then dissociates to give ammonium and hydroxyl ions. This behavior of glycine may be represented as



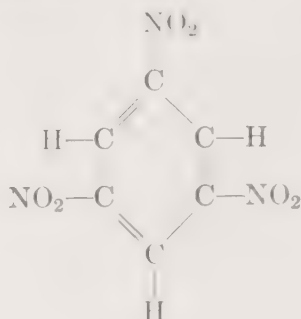
Glycine could react with an acid such as hydrochloric with the formation of an amino acid hydrochloride and water. At a hydrogen-ion concentration specific for each amino acid, the amino acid would be neutral, reacting equally as an acid or a base. The hydrogen-ion concentration at which an amino acid or protein is neutral is known as the *isoelectric point* of the substance.

The fact that proteins consist of amphoteric amino acids is of great importance in interpreting their reactions. The amino acids are linked together to form the protein molecules, but there are free amino ( $-\text{NH}_2$ ) and free carboxyl ( $-\text{COOH}$ ) groups on the protein molecule, and these groups can react in a manner similar to that pictured above for the simplest amino acid.

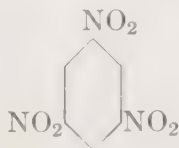
**Dyes.** A dye may be considered as a colored organic compound which is capable of combining with a wide variety of substances and imparting color to them. In order for a compound to act as a dye, it must contain what are known as *chromophore* and *auxochrome* groups linked to benzene rings. A chromophore group imparts the color to the molecule of the dye while the auxochrome group imparts the property of electrolytic dissociation to the molecule, thereby making it more reactive. This can be illustrated by the following example: Benzene is a colorless molecule, but when three of the hydrogen atoms in the molecule are replaced with



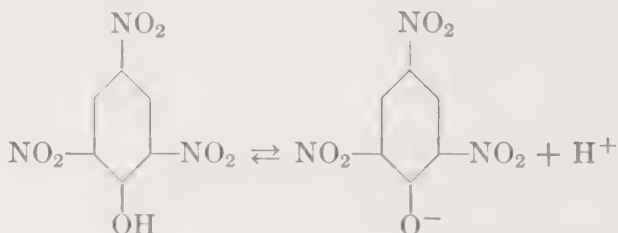
nitro groups (chromophores), a colored compound, or chromogen, is produced of the formula



which can be more simply represented as



Trinitrobenzene is yellow, but it is not a dye because it does not dissociate to any particular extent and hence is unable to form salts with either acids or bases. When another hydrogen atom is replaced with a hydroxyl group, the colored compound picric acid is formed, and it is capable of dissociation in the following manner:



Hence the hydroxyl group in picric acid is the auxochrome group of the dye picric acid. The dye ion has a negative charge as indicated in the above formula and is known as an *acidic dye* because it is capable of forming salts with bases. Auxochrome groups may be either acidic or basic, and a dye possessing a basic auxochrome group such as  $-\text{NH}_2$  is known as a *basic dye* since it reacts as a base with an acid to form a salt. Both acidic and basic dyes are employed for staining bacteria. The majority of the bacterial stains are prepared from basic dyes, as they react more readily with bacteria in neutral or alkaline solution. Unfortunately the use of acidic dyes is too often neglected. While it may be more difficult to stain bacteria with acidic dyes, yet the stained preparations are often much sharper when the latter type of dye is employed.

The addition of a variety of substances to staining solutions causes the bacteria to stain more deeply than they would in the absence of the added substance. Substances which enhance the staining ability of a dye are spoken of as *intensifiers*. Basic dyes stain bacteria more readily if the alkalinity of the dye solution is increased, while the reverse holds true with the acidic dyes. Acids, bases, wetting agents (or detergents), aniline, and phenol are examples of intensifiers commonly employed with bacteriological stains. The application of heat to the staining solution will in many instances increase the rate and possibly the extent of staining; hence heat may be regarded as a physical intensifier. In the staining process, whether of cloth or paper or of tissues or cells, chemicals are sometimes employed which have a strong affinity for both the dye and the substance being stained and thus bring about a firmer union between the dyestuff and the substance being stained. Substances which enhance the strength of the union between dye and substrate are called *mordants*. Typical mordants employed in staining reactions are tannic acid in the *flagella stain* and iodine in the *gram stain*.

**Mechanism of Staining.** The retention of dyes by cells has been explained primarily on the basis of either a physical union or a chemical union between the dye and components of the cell. According to the physical concept of the staining reaction, deposition and retention of the dye occur on surfaces as a result of forces primarily physical in nature. Staining is pictured as an adsorption reaction which may occur in variable proportions and in which no new compound of definite chemical composition is formed. Proponents of the chemical theory postulate that a reaction occurs between the dye and the material being stained with the formation of reaction products of definite composition and that the reaction takes place in *stoichiometric proportions*.

As a result of a chemical reaction, a new compound is formed with properties different from those of the reacting substances. Furthermore, simple washing with water or other solvents generally does not decompose the compound so formed with the liberation of the original reactants. There is little evidence that the bacteria react with a dye to form new chemical substances, and it is usually possible to extract the dye from the cells by prolonged immersion in water, alcohol, or other solvents. On the other hand, it might be argued that a mass-action effect controls the staining reaction and that the dye-cell complex dissociates in the solvent with the liberation of unstained cells and the free dye. In many of the quantitative relationships of the staining reaction the results are, however, in general agreement with those postulated on the basis of an adsorption reaction. The controversy between the proponents of the physical and the chemical theories of the staining reaction loses much of its meaning when staining is considered in the light of modern colloidal chemistry.

When bacteria are placed in an electrical field, they will migrate toward one of the electrodes. The direction of their migration is dependent on the nature (+ or -) of the over-all electrical charge possessed by the bacteria, i.e., on the difference between the total positive and negative charges at the surface of the cell. Bacteria in suspension on the alkaline side of their isoelectric point possess a negative charge and migrate toward the positive pole, behaving in this respect like anions. We can picture the bacterial surface as a mixture of acidic and basic groups with the acidic groups predominating under physiological conditions of hydrogen-ion concentration. This predominance of negative groups at the cell surface results in the attraction of positively charged ions to the vicinity of the cell, and a layer of cations will surround the cell. When the cells are transferred to a new ionic environment, an exchange of ions will occur with the formation of a new ionic layer in order that equilibrium may be established with the new environment. McCalla has shown that bacteria will absorb various ions when the ionic environment is changed, e.g., magnesium ions will be absorbed when the cells are suspended in a solution of a magnesium salt. This reaction may be represented as



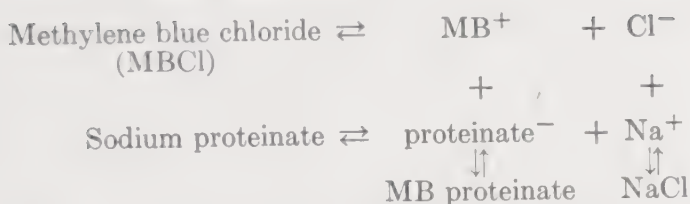
where  $B$  represents the bacterial cell and  $n$  an unknown number of negative charges carried by the cell.

When a second positively charged ion is added to the suspension, the added ion will compete with the magnesium for a place around the cell and may replace it entirely. When a basic stain such as methylene blue is added to the suspension, the positively charged dye ion will compete with the magnesium or other positively charged ions at the surface of the cell and may replace them by means of an ionic exchange reaction. This reaction can be represented as

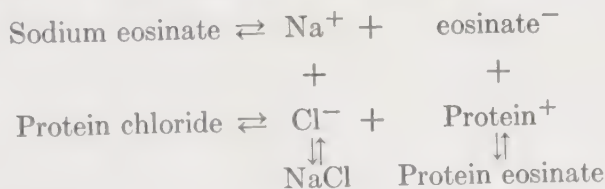


if the reaction goes to completion. McCalla considers that the reaction of stains with bacteria can be interpreted as an ionic exchange reaction which approaches stoichiometric proportions.

The great variation in the reactivity of the components of bacteria and the relatively unknown behavior of dyestuffs at the complex surfaces of the cell render it dangerous to attempt to simplify the complexity of reactions which enter into the staining process. For our purposes, it will suffice to bear in mind that staining is a highly complex reaction which, however, may be pictured as ionic exchange reactions in the following manner:



when the reaction occurs between a basic dye and bacterial proteins ionizing in alkaline solution as an acid. On the other hand, the reaction in acidic solution with an acid dye may be pictured as



These equations are not offered as an explanation of the staining reactions of bacteria but simply as a means of visualizing what may occur when bacteria are treated with a dye. The process may be physical or chemical in nature, or it may involve a combination of both physical and chemical factors.

### DIFFERENTIAL STAINS

**The Gram Stain.** The differential staining technique is generally more difficult than simple staining and is for the purpose of differentiating between different parts or structures of the cell or between different groups of organisms. The gram stain is the most important differential stain employed in bacteriology and serves to differentiate bacteria into two main groups: the *gram positive* and the *gram negative*. Gram observed in 1884 that in tissue sections stained with gentian violet and then treated with a dilute solution of iodine in potassium iodide solution, the stain could be readily removed from the tissue by alcohol but not from most bacteria within the tissue. Gram also observed that when this procedure was applied to smears of various bacteria, certain species retained the original dye while others were rapidly decolorized by the alcohol and would take up another stain. These observations were soon confirmed, and it was found that bacteria can be divided into two general groups, *gram positive* and *gram negative*, on the basis of their behavior in the gram stain. This division of bacteria into two groups on the basis of gram-staining properties is of great value in the identification of many bacteria, being particularly important with certain of the animal pathogens, e.g., gram-negative and gram-positive cocci.

There have been some improvements in the technique of carrying out



the gram stain, but basically the procedure remains the same as in the original method. Let us assume that we have a mixture of bacteria on a slide and that they are stained with a pararosaniline dye such as crystal violet. All cells will be colored a dark violet, for they all stain much alike with this basic stain. Then we rinse off the excess dye and treat the smear with iodine solution, the iodine forming a deep-blue-black complex with the crystal violet and tending to mordant the dye to the cells. The iodine solution is poured off the slide, which is then immersed in alcohol or an alcohol-acetone mixture until no more of the dye is *readily* removed from the smear, a procedure generally requiring from 10 to 30 sec. This decolorization is the most important step in the gram stain technique, for alcohol or acetone removes the dye complex from gram-negative bacteria *much more rapidly* than from gram-positive forms. Gram-positive cells can be decolorized if decolorization is prolonged. When decolorization has been properly carried out, the gram-positive cells will still be colored bluish black while the gram-negative bacteria will be colorless. In order to observe the colorless cells more readily, it is necessary to stain them with a basic dye of a color different from crystal violet. The gram-positive forms, being nearly saturated with dye, do not take up any appreciable amount of the counterstain, while the gram-negative cells are stained by the second dye and assume the color of this dye.

The ability of gram-positive bacteria to retain the original dye is by no means absolute, and there are varying degrees of gram positivity among the different gram-positive species. Furthermore, the results of a gram stain depend to a considerable extent on the proper preparation of the smear and a standardized technique of carrying out the staining procedure, as well as upon the nature of the cells, their age, and environmental factors, particularly pH. Gram-positive cells on death tend to become gram negative, and this tendency also is observed with increasing age of the bacteria. On the other hand, there is little or no tendency for gram-negative cells to exhibit gram-positive staining characteristics.

The ability of cells to retain the original stain in the gram stain is not a property characteristic of cells in general but is confined almost entirely to certain species of bacteria, to the yeasts, and to the molds. The molds tend to stain irregularly in the gram stain while the majority of other plant and animal cells are readily decolorized and take up the counterstain, i.e., they are gram negative.

Gram's stain, when properly employed, is one of the most important tools of the bacteriologist. However, the gram stain is something more than an aid in the identification of bacteria, since it indicates a fundamental difference between the two groups of bacteria. While there are exceptions, gram-positive organisms tend to react with many agents in a manner different from and frequently the reverse of the reaction exhibited

by the gram-negative forms. Certain of these differences are best expressed in tabular form as in Table 2-2. It must be remembered that

TABLE 2-2

	Gram positive	Gram negative
Isoelectric staining point.....	pH 2 to 3	pH 5
Digestion by pepsin or trypsin.....	Resistant	Not resistant
Solubility in 1% KOH.....	Not soluble	Soluble
Growth in acidic media.....	Inhibited	Less inhibited
Resistance to mechanical agents.....	Resistant	Less resistant
Bacteriostatic action of triphenylmethane dyes.....	Marked	More resistant
Inhibition by sodium azide.....	Resistant	Less resistant
Susceptibility to antibiotic agents.....	Pronounced	Less susceptible
Ability to stimulate antibody formation.....	Poor antigens	Good antigens
Undergo autolysis.....	Resistant	Less resistant

the differences between gram-positive and gram-negative bacteria are frequently quantitative rather than qualitative in character and that there are exceptions to all the statements in Table 2-2. This tabulation expresses *tendencies* rather than absolute differences.

Because of the differences between these two groups of bacteria, many studies have been carried out in an attempt to explain the basic differences, or at least to gain further insight into the mechanism of the gram stain. There are numerous observations which suggest that gram positivity depends upon the intactness of the cell wall, since gram-positive bacteria upon physical disintegration exhibit staining properties characteristic of the gram-negative forms, i.e., bacterial protoplasm stains gram negatively. Some workers claim that this behavior indicates that permeability of the cell is the controlling factor in the gram stain, the iodine-dye complex readily diffusing through the cell membrane of gram-negative forms while it diffuses with difficulty from the gram positives. Others claim that the integrity of the cell wall is essential for the maintenance of surfaces involved in the staining reaction and that the gram stain is dependent upon physical or chemical reactions at these surfaces.

Recent studies by Henry and Stacey and by Bartholomew and Umbreit suggest that a magnesium ribonucleate apparently relatively abundant in the surface of gram-positive organisms is responsible for the particular staining property of these bacteria. When the magnesium ribonucleate is removed by chemical or enzymatic means, gram positivity is lost, but it can be regained by "replating" the magnesium ribonucleate on the gram-negative skeleton of originally gram-positive cells. True gram-

negative cells cannot be converted to gram-positive forms by simple treatment with the ribonucleate, and the ribonucleate by itself is not gram-positive. Recent studies, however, indicate that gram-negative bacteria (*Escherichia coli*) can be plated with a gram-positive coat if a highly viscous solution of ribonucleate is employed. These observations suggest that gram positivity is associated with a protein-magnesium ribonucleate complex in the cell surface. Certain contradictions still exist between the results of different studies on the nature of the gram stain, and further work is needed before it will be possible to determine the nature of the basic differences between the two great groups of bacteria.

**Acid-fast Stain.** The property of acid fastness, i.e., resistance possessed by certain bacteria to decolorization of their stained cells even by mineral acids, was first noted in 1882 by Ehrlich in studies on the tubercle bacillus. The mycobacteria and a few strains of diphtheria-like bacteria and actinomyces are stainable with difficulty by the ordinary stains, but once stained they retain the dye quite tenaciously. They can be stained if a concentrated solution of a dye such as basic fuchsin is employed in the presence of phenol acting as an intensifier. The rate of staining is slow at room temperature, but staining can be accomplished in a few minutes at a temperature near the boiling point of water. The Ziehl-Neelsen technique, frequently employed for the staining of acid-fast bacteria, takes advantage of these facts. Once stained, acid-fast bacteria retain the dye even on prolonged immersion in water or alcohol acidified with a mineral acid while non-acid-fast cells are decolorized in a few seconds.

When a mixture of acid-fast tubercle bacilli and non-acid-fast forms such as might be encountered in tubercular sputum is stained by the Ziehl-Neelsen technique, all the bacteria will take up the original stain. On treatment with acid alcohol, the non-acid-fast forms will be decolorized and can subsequently take up a counterstain. Hence, the acid-fast bacteria can be readily differentiated from the non-acid-fast bacteria on microscopic examination, appearing as reddish cells while the non-acid-fast bacteria take on the color of the counterstain.

In one modification of the acid-fast stain, a detergent (wetting agent) is added to the staining solution, and the acid-fast forms are stained in a few minutes at room temperature. Another modification of the staining method employs auramine as the dyestuff because it possesses the property of fluorescence. When auramine-stained cells are illuminated with ultraviolet light, they emit light of a longer wavelength, to which the eye is sensitive. Non-acid-fast bacteria stained with auramine are decolorized with acid alcohol while the acid-fast bacteria retain the auramine. The smear is examined with a microscope equipped for ultraviolet-light illumination and with a yellow filter to remove blue light entering the



ocular. The field of view appears dark except for the auramine-stained, acid-fast bacteria, which stand out as luminous yellow bodies in the dark background.

Acid fastness was explained for many years on the basis of a high concentration of fatty or waxy material in the acid-fast forms. In recent years it has been demonstrated that acid fastness of the mycobacteria (e.g., *Mycobacterium tuberculosis*) is not dependent on the total fat content of the cells but appears to be due to a specific component, mycolic acid, present in the waxy material. Mycolic acid, while somewhat acid fast by itself, appears to be in combination with a polysaccharide in the cell, and this combination may possess stronger acid-fast properties than mycolic acid alone. The possession of mycolic acid by all acid-fast forms has not been demonstrated, and it is possible that acid fastness is the result of the possession of other acid-fast-staining materials or even of particular cell membrane structures. Our knowledge of some of the simpler facts concerning bacteria and other microorganisms is still far from being complete.

**The Spore Stain.** Bacterial endospores stain like the acid-fast bacteria and will retain the primary stain in the Ziehl-Neelsen stain while the originally *vegetative* portion (spore case or sporangium) of the cell is non-acid-fast. The Ziehl-Neelsen stain can be employed for differentiating between the spore and the sporangium or for the observation of free bacterial spores, since washing with water is ordinarily sufficient to decolorize the spore case. Treatment with acid alcohol may remove the dye from the spore and should be omitted. In general, spores are more readily stained than are acid-fast bacteria, and quite frequently an aqueous solution of malachite green is employed as the primary stain, although heat is necessary to secure good staining. The excess stain is rinsed off with water and the cells counterstained with eosin or safranin. Low permeability of the spore membrane to dyestuffs is the most common explanation of the peculiar staining property of bacterial spores (see Fig. 2-9). It should be pointed out at this time that endospores can be observed in ordinarily stained preparations as colorless bodies within the stained portion of the original cell, and that a thin film of dye may be deposited on the exterior of free spores.

**Granules.** In many bacteria stained by ordinary methods, it is possible to see bodies or granules stained more intensely than the rest of the cell. When the granules assume a color different from the rest of the cell, they are spoken of as metachromatic granules. The composition and function of these granules are still matters of debate, although in many instances the granules may be reserve food materials of various sorts which are stored in the cells. They tend to accumulate as growth slows down and to disappear again when the cells are actively growing.



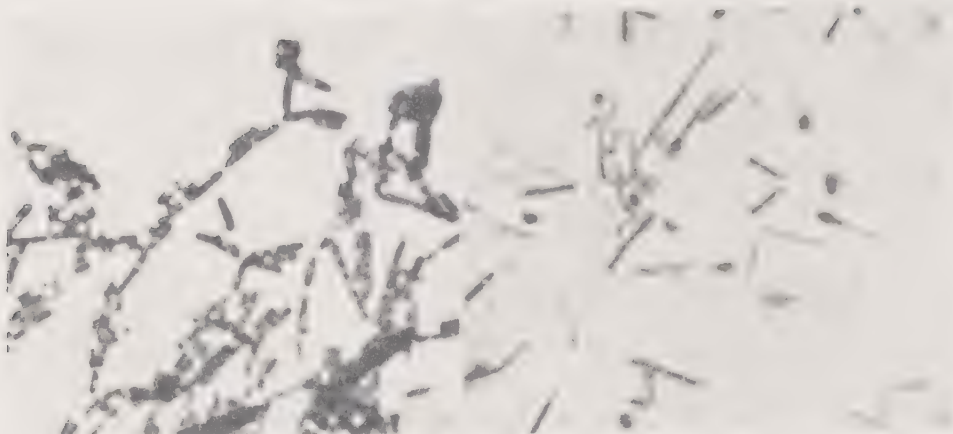


FIG. 2-9. Crystal violet stained preparation of (A) *Bacillus cereus* (spores unstained) and (B) malachite green-safranin spore stain of *Clostridium tetani* (spores dark).

These granules may be composed of fats, carbohydrates, or complex nitrogenous matter, and in the thiobacteria granules of inorganic sulfur can be present. Fat globules are present in a wide variety of microorganisms and can be recognized as highly refractile bodies which are not stained by the ordinary stains. Fat globules can also be recognized by the fact that they stain black when the cells are treated with osmic acid or will take up fat-soluble dyes such as Sudan III, the bulk of the cell not being stained.

The reserve carbohydrates appear to be of two general types, glycogen and granulose. The first is recognized by the reddish-brown color which develops on treatment with iodine solution and is considered to be the same as or similar to the glycogen found in tissues such as the liver. It has not been positively identified in all bacteria showing reddish-brown granules after iodine treatment, and hence it might be better to consider these granules as glycogen-like bodies. The glycogen granules have often been mistaken for nuclei but can be differentiated by the fact that they are not removed from the cell by boiling water and can be destroyed on hydrolysis with mineral acids. The second type of reserve carbohydrate granules gives a blue color with iodine and is chemically related to starch. Granulose is not as widely distributed in the bacteria as is glycogen. Both types of granules are visible in the dark field.

Very little is known about the nitrogenous granules except for volutin, a reserve material which is found in the cells of a wide variety of microorganisms. Volutin granules can be observed in living cells under the dark field or in photographs of bacteria taken with the aid of the electron microscope. Volutin has a strong affinity for basic dyes, and volutin granules can be observed as more intensely stained bodies within cells stained by the ordinary techniques. When the cells are stained with an



FIG. 2-10. Metachromatic granules in *Corynebacterium diphtheriae*.

old solution of methylene blue, the volutin granules exhibit a color different from that of the bacterial protoplasm, volutin strongly adsorbing the methylene violet present in old solutions of methylene blue. This metachromatism is responsible for the name *metachromatic granules*. Metachromatic granules are generally more resistant to decolorization than the bulk of the cell, and there are a number of differential stains for metachromatic granules based on this behavior. If volutin-containing bacteria such as diphtheria bacilli, which are particularly rich in volutin, are stained with methylene blue and counterstained with Bismarck brown, the latter dye will replace the methylene blue in the cytoplasm and the granules will then appear as blue-black bodies in a yellowish-brown cytoplasm. Volutin can also be stained (vital stain) in the living cell with dilute methylene blue or with neutral red. Volutin appears to be composed of zymonucleic acid, possibly in combination with proteins or organic bases, and it has been suggested that it serves as reserve building material for the rebuilding of certain constituents of the enzymes of the cell. Volutin granules have frequently been mistaken for nuclei, and the nucleoprotein of these bodies is closely related to the nucleoproteins found in the true nucleus of other cells. Recent studies suggest that the staining properties of these granules are due to the presence of metaphosphates, the latter inducing polymerization of the dye with resultant color change or metachromatism.

The amount of reserve food material formed by the cell and stored as discrete particles varies considerably with cultural conditions. Formation of granules is usually limited when the cells are cultivated in a poor medium while the presence of carbohydrates tends to stimulate the deposition of fat and of carbohydrate granules. Information regarding the nature of the reserve food materials is of some value in identifying different species of bacteria, particularly the aerobic sporeformers.

**Flagella Stains.** With most bacteria, the flagella are so minute as to be invisible on microscopic examination. Flagella have been observed on dark-field examination of certain species of bacteria, but their presence generally is recognized by the fact that a particular cell is motile. The presence of flagella can be demonstrated by special staining techniques which involve the deposition of sufficient dye on the flagella to create a body large enough to be resolved under the microscope. The staining processes employed for this purpose may also increase the intensity of staining of the individual flagella sufficiently that those near the lower limits of resolving power become apparent without marked increase in thickness, or both processes may be involved.

The basis of all the flagella stains is a preliminary treatment of the cells with a mordant, which is generally a complex colloidal solution frequently containing tannates. The success of the stain depends to a considerable extent on the colloidal state of the mordant. Flagella are extremely fragile, and great care must be employed in handling the organisms and in the preparation of the smear. The presence of organic matter and of cellular debris generally hinders the demonstration of



FIG. 2-11. Flagella stain of *Salmonella typhosa*.

flagella, as the foreign matter may react with the mordant and adsorb considerable amounts of the dye, thus interfering with the observation of the smear. Flagella can also be observed following the deposition of silver rather than of dyestuff on the mordanted preparation.

**Capsule Stains.** A number of bacterial species under favorable conditions develop an enclosing sheath, or envelope, which is called a capsule. In most cases the capsule is a layer of gelatinous or gummy material which is not stained readily with the ordinary stains. Capsules can be demonstrated as a somewhat more transparent layer around the cells in negatively stained preparations. To increase the contrast between the cell and its capsule, a negatively stained smear can be treated with a



FIG. 2-12 Negatively stained preparation of *Klebsiella pneumoniae* showing capsules

regular stain, which will react with the cell proper, leaving a stained cell surrounded by a clear zone in the background created by the negative stain. When Congo red is employed as the negative stain, the cells can be stained with an acidic dye in acid solution, the acid at the same time tending to fix the Congo red to the slide in its insoluble acid form. Capsules can also be demonstrated as faintly stained halos around cells stained with gentian violet or carbolfuchsin if the time of staining is prolonged and the excess stain is removed by blotting. Capsules are generally more pronounced around pathogenic forms in slides prepared directly from body fluids, capsule formation being enhanced in the body. The demonstration of capsules is also facilitated by the proteinaceous material present in body fluids. This forms a film of material in the smear which acts somewhat like the background in a negatively stained preparation.

**Cell-wall Stain.** Cytoplasmic membranes and other structures can at times be observed directly either in a bright-field or in a dark-field prepa-



ration of bacteria. Knaysi developed a staining procedure which is of value in demonstrating the cell wall and the slime layer around bacteria. It consists of mordanting a heat-fixed smear of bacteria with a mixture of tannic acid and alum and staining with a drop of Ziehl-Neelsen carbolfuchsin under a cover glass (see Fig. 3-5). The cytoplasm appears dark red in cells stained by this method while the cytoplasmic membrane is still darker; the cell wall stands out as a blue structure, and the slime layer is bright red. Considerable differentiation can also be observed when bacteria are examined directly in suspension in dilute (1:20,000)

crystal violet (gentian violet) in a hanging drop or in a film under a cover slip (see Figs. 1-11 and 2-13).

**Differential Staining during Growth.** Before we leave consideration of the principles of staining bacteria, it might be well to consider an application of the staining reactions to bacteria in actively growing cultures. Pathogenic bacteria of the enteric group, *Salmonella typhosa* (the typhoid bacterium) being a good example, generally are unable to ferment lactose. However, this sugar is readily fermented by closely

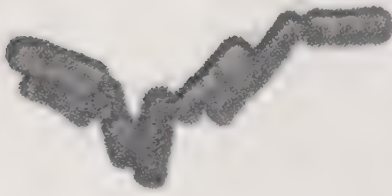


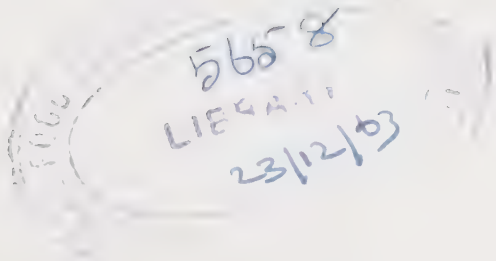
FIG. 2-13. Photomicrograph of *Bacillus subtilis* suspended in a dilute solution of crystal violet.

related, nonpathogenic forms such as *Escherichia coli*. Since pathogenic bacteria of the enteric group are associated with coliform bacteria (*E. coli* and other bacteria closely resembling it) in fecal matter and as these organisms are culturally and morphologically similar, it is difficult to detect the presence of the pathogenic forms in ordinary streak cultures on nutrient agar. Lactose can be incorporated in nutrient agar along with two dyes, eosin and methylene blue. These dyes, one acidic and the other basic, unite to form a slightly acidic complex which is unable to stain the cells at the approximately neutral pH of the lactose agar. The coliform bacteria ferment the lactose with the production of considerable amounts of acid, and in an acidic environment some of the eosin-methylene blue complex is taken up by the bacteria, the faintly stained cells giving rise to colored colonies. The non-lactose-fermenting bacteria do not produce acid, and their colonies remain colorless. The colored colonies of lactose-fermenting bacteria which develop on eosin-methylene blue agar can be eliminated as possible pathogens, and one can proceed to an examination and identification of typical colorless colonies of non-lactose-fermenting bacteria as possible pathogenic forms.

With these pertinent facts concerning the staining reactions as a background, we can employ stains more intelligently in the laboratory and proceed to a more direct consideration of the structure of the bacterial cell.

#### REFERENCE

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## CHAPTER 3

### THE STRUCTURE OF BACTERIA

To appreciate the difficulties involved in cytological studies of bacteria, it is necessary only to recall the minute size of these microorganisms. The majority of bacteria have diameters of 0.5 to 1  $\mu$ , and since the limit of visibility in the ordinary microscope is around 0.15  $\mu$ , structures within the cell are approaching, or may be even less than, the limits of resolving power. Size is not the only difficulty in the study of the structure of bacteria; the fact that the cytoplasm of bacteria is usually nearly optically homogenous makes observation even more difficult. It is therefore desirable to be able to stain a given structure in such a way as to obtain maximum contrast between the structure and the remainder of the cell. In many instances staining procedures are not sufficiently selective, and confusion results in the interpretation of the observations, granules being identified as nuclei or as structures other than what they actually are. Despite the difficulties encountered in the study of the structure of bacteria, there is a surprising amount of data on this subject. In addition, the concepts of the structure of bacteria are based in part on reasoning from observations on the structure of larger cells.

Living matter consists of units of protoplasm called *cells*. Early observers, when reporting their observations of living matter, presented drawings containing structures which might be regarded as cells, but it was not until 1665 that the existence of "little boxes or cells distinguished from one another" was definitely reported by Hooke. It is now recognized that living matter exists in cells varying widely in size and shape, but in all the higher cells three major parts can be differentiated—an *outer wall* surrounding the *cytoplasm*, or cell body proper, within which is located the *nucleus*. Other structures may be present within the cytoplasm or attached to the cell, but they are not characteristic of all cells.

**General Cytology of Bacteria.** The bacterial cell consists of *cytoplasm* enclosed within a *cytoplasmic membrane*, which presses against the *cell wall*, which in turn is surrounded by a *slime layer*. Certain bacteria, particularly those of the genera *Bacillus* and *Clostridium*, are able to form a

single spore per cell. These endospores are generally more resistant to unfavorable conditions than are the vegetative cells, frequently withstanding boiling water for an hour or longer, vegetative cells being killed in a matter of seconds. The motile bacteria have one or more filamentous appendages, flagella, which serve as organs of locomotion but which are too thin to be resolved under the ordinary microscope. In addition to these structures, the bacterial cell may contain vacuoles, pigment-bearing bodies, fat globules, or granules, but these bodies together with endospores and flagella are specializations not common to all bacteria. The structure of a hypothetical bacterium is indicated in Fig. 3-1, but it should be emphasized that this figure is a schematic one. The existence of such structures is based upon evidence to be described and suggested in some of the following figures. The major structures are evident in the electron micrographs of *Bacillus mycoides* (Fig. 3-2) and of a cross section of *Bacillus cereus* (Fig. 3-3). Recent studies on the cytology of bacteria have been reviewed by Knaysi (1956) and discussed in more detail by Bisset (1955).

When unstained bacteria are examined under the microscope they appear for the most part as simple, undifferentiated, homogenous masses of protoplasm, although each species generally possesses a fairly uniform size and shape. When the cells are stained very lightly

with basic aniline dyes, one can at times observe a darker line at the surface of the cytoplasm. When these cells are examined under the dark field, they appear to be surrounded by a luminous line around the more or less dark interior of the cell. This luminous line is due to reflection of light by the denser outer layer of the cytoplasm, which also binds dyestuff to a greater extent than does the cytoplasm.

Physicochemical principles lead to the conclusion that protoplasm, being different in composition from the environment, must possess a surface or interface differing at least in concentration of materials from the

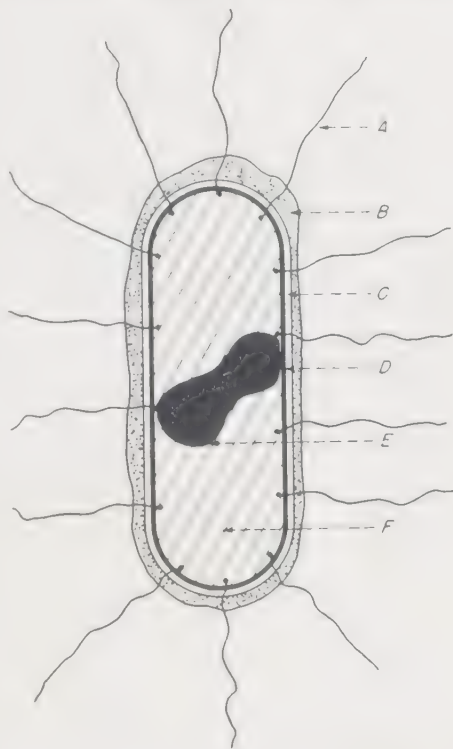


FIG. 3-1. Schematic representation of the structure of a bacterium. (A) flagella, (B) slime layer, (C) cell wall, (D) cell membrane, (E) nucleus, (F) cytoplasm.





FIG. 3-2. Electron micrograph of *Bacillus mycoides* showing (A) slime layer, (B) cell wall, (C) cell membrane, and (D) cytoplasm. The unevenness of these structures is due in part to uneven shrinking during drying. [From Knaysi and Barker, *Journal of Bacteriology*, **53**, 541 (1947).]

bulk of the cytoplasm, since such a condition always exists at the boundary between any two systems or phases. This leads to the assumption of the existence of a cell membrane, although it may consist of nothing more than a denser layer of protoplasm in which the molecules are oriented in a definite manner. This membrane may be in immediate contact with a definite, protective cell wall. The terms cell wall and cell membrane have often been used indiscriminately, and some investigators have simply considered bacteria to be composed of two main parts—an inner *endoplasm* and a narrow, denser outer zone, the *ectoplasm*. The cell membrane from this viewpoint would probably be considered as a constituent part of the endoplasm and the outer layers or attachments as part of the ectoplasm. The terms *cytoplasm*, *cytoplasmic membrane*, *cell wall*, and *slime layer* as defined by Knaysi have the merit of being definite, experimentally demonstrable structures and of expressing properties characteristic of

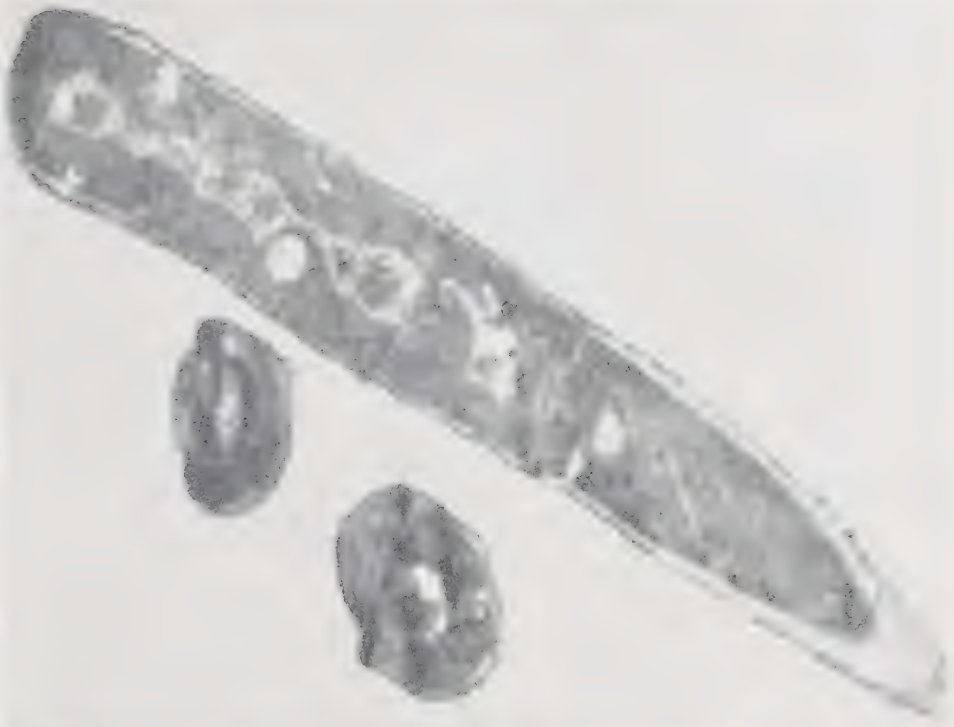


FIG. 3-3. Electron micrograph of a thin longitudinal section of *Bacillus cereus*. [From Chapman and Hillier, *Journal of Bacteriology*, **66**, 363 (1953).]

such biological structures. These terms will be employed in the discussion to follow.

**Cytoplasm.** Cytoplasm is that material which occupies the volume of the cell bounded by the cell wall and membrane, and in which are located the granules, spores, vacuoles, and other internal bodies characteristic of the species. It is an exceedingly complex colloidal system or systems composed of water, proteins, fats, carbohydrates, and inorganic matter in a wide variety of combinations. When free from inclusions, cytoplasm is generally optically and electronically homogenous and stains uniformly when treated with solutions of either acidic or basic dyes. Yet the chemical events occurring within the cell must be coordinated in time and space to permit of orderly metabolic activity and growth. It is therefore reasonable to conclude that cytoplasm does possess a structure at least sufficient to maintain the different enzyme systems in juxtaposition, and possibly there is a well-defined, although submicroscopic, morphological arrangement of these biochemical units. Cells of the larger bacteria can be crushed under the microscope, and the protoplasm can be observed to flow out of the cell as a semiliquid substance. There is evidence that a

membrane forms around the exuded material, and this self-forming membrane could be analogous to that surrounding the cytoplasm of the intact cell. Differential centrifugation of such material or of cytoplasm from mechanically disrupted cells indicates the presence of particulate matter, generally of submicroscopic size, in cytoplasm. Some workers support the concept that bacteria contain mitochondria, bodies which in higher cells are particularly rich in the terminal respiratory enzymes and systems. The postulated existence of mitochondria in bacteria is based upon the observation within the cells of areas which appear to be particularly active in the reduction of the tetrazolium dyes which are indicators of oxidation-reduction activity. Bisset (1955) does not support this concept but does suggest that there are marked accumulations of nucleoproteins at the "growing point" of bacteria. An increase in concentration of enzymes at such points might occur and give rise to what appears to be mitochondria in tetrazolium-treated cells. Additional evidence is needed before it can be stated definitely that bacteria contain mitochondria, or microscopic equivalents thereof.

**Cytoplasmic Membrane.** The cytoplasmic membrane tends to stain more deeply with basic dyes than does the cytoplasm and tends to retain the stain to a greater extent than the cytoplasm on decolorization in slightly acidic solutions. The cytoplasmic membrane may result from a condensation and orientation of cytoplasmic constituents at the surface of the cytoplasm, surface-active materials such as the lipoproteins in particular tending to concentrate at this interface. Increased lipoprotein and high nucleoprotein content may account in great part for the staining properties of this structure, the highly acidic lipoproteins and nucleoproteins forming a firmer union with basic dyes than the less acidic proteins making up the bulk of the cytoplasm.

When one immerses cells of higher plants in concentrated salt solutions with osmotic pressures greater than that of the protoplasm, water passes out of the cell and the cytoplasm shrinks away from the cell wall. This behavior, known as *plasmolysis*, reveals clearly the existence of a cell membrane distinct from the cell wall. The same phenomenon has also been demonstrated with some of the larger bacteria and can be observed in electron micrographs of bacteria (Fig. 3-4). The turgor, or osmotic pressure, of the cytoplasm of cells in their normal environment is sufficient to press the membrane tightly against the cell wall, thus making it more difficult to distinguish between the cell wall proper and the cytoplasmic membrane.

The cytoplasmic membrane, possibly together with the cell wall to a limited extent, controls the entrance of food-stuff into and of waste products out of the cell. Little is actually known of the nature of the cell membrane and of how it acts. There is evidence that it is a changing



FIG. 3-4 Electron micrograph of *Serratia marcescens*, illustrating different degrees of shrinkage of the cytoplasm from the rigid cell wall. [From van Hese, *Biochimica et Biophysica Acta*, **1**, 529 (1947).]

structure and that permeability of the cytoplasmic membrane can alter with age of the cells, young cells in general appearing to be more permeable to both foodstuffs and to inhibitory agents than older cells. Permeability of this membrane can also change with changes in the nature of the environment and thus serves as a regulatory barrier for the maintenance of a dynamic equilibrium between the cytoplasm of the cell and the fluid with which it is in contact. Hence it can be termed the osmotic barrier.

The actual demonstration of the cell membrane as a structure has been accomplished in lysozyme digested bacteria. Under carefully controlled conditions the cell wall can be dissolved away, leaving fragile protoplasts which are metabolically active. In the last stages of dissolution the protoplasts may appear as "ghosts," the empty membranes surviving momentarily.

**Chemical Composition.** Since cytoplasm makes up the great bulk of most bacteria, chemical analyses of bacteria as a whole give results primarily indicating the nature of the protoplasm. The bacterial cell is similar in chemical composition to cells of other species of plants and animals. Water is the chief constituent, amounting to 75 to 85 per cent of the total fresh weight. The solid matter is composed of 70 to 95 per cent organic matter and 30 to 5 per cent inorganic matter. The average carbon content of the organic matter is about 50 per cent, while nitrogen in organic combination constitutes 5 to 10 per cent of the organic content of the cells. The concentrations of the various organic constituents vary



to some extent with the nature of the organism and with the nature of the environment in which the cells developed.

The inorganic constituents of the bacterial cell show greater differences in relative amounts than do the organic compounds, which enter more intimately into the structure of the cell. A portion of the inorganic matter is in chemical union with the organic matter, but the great bulk of the salts appears to be present in solution, maintaining ionic and osmotic equilibrium between the cell and its environment. The main inorganic elements found in the cell are phosphorus (2 to 32 per cent), sulfur (0.2 to 9 per cent), sodium (0.2 to 20 per cent), potassium (2 to 38 per cent), calcium (0.05 to 11 per cent), magnesium (0.1 to 13 per cent), iron (trace to 6 per cent), and chloride (trace to 40 per cent). Minute amounts of other elements such as molybdenum, vanadium, copper, zinc, or boron may be essential for some particular activity of the cell, and these trace elements are generally present as impurities in the salts employed in culture media.

**Reserve Materials.** While the cytoplasm of many species of bacteria appears to be more or less homogenous, there are a number of species in which material is deposited within the cell in vacuoles or as free granules. These bodies tend to appear in the cell as multiplication slows down or ceases and to disappear when the cells are rapidly multiplying or are under conditions of prolonged starvation. This suggests that these bodies are an accumulation of reserve food material, and chemical analyses have demonstrated that they are composed of fat, carbohydrate, or nitrogenous material, or of sulfur or calcium carbonate in the case of certain autotrophic species. These granules have been mentioned in the previous chapter and are of importance in the economy of the cell but appear to have no other function than as a store of reserve food material. When these reserve food materials are water-soluble, water can collect around them with formation of a vacuole containing reserve material in solution.

**The Cell Wall.** The cytoplasm and its membrane occupy a volume limited by the cell wall, which imparts rigidity and shape to the bacterial cell. The cell wall may be observed as a thin structure around plasma-lyzed cells, either in the light microscope or better in electron micrographs or in cells stained by suitable procedures (see Figs. 3-3 to 3-5). In ordinary stained preparations the cell wall is not visible, because the small amount of dye absorbed during the staining process is washed out when the smear is rinsed with water. When the cells are observed in suspension in a solution of methyl violet, the cell wall appears as a faint purple line in contrast with the darker violet cytoplasm which it encloses. In the preceding chapter we considered a method of producing contrast between the structures, mordanting the cells with a mixture of tannic acid and alum and observing them covered with carbolfuchsin under a cover



FIG. 3-5. Cell-wall stain of *Bacillus cereus*. (From Knaysi, "Elements of Bacterial Cytology," Comstock Publishing Associates, Inc., Ithaca, N.Y., 1944.)

glass. In such a preparation the cytoplasm is dark red while the cytoplasmic membrane is still darker and is in immediate contact with the cell wall, which stains blue and is surrounded by a bright-red slime layer.

The chemical composition of the cell wall varies in different species of bacteria and in many species appears to be composed of cellulose or of hemicellulose. In other species the cell wall contains nitrogenous matter, some studies suggesting that it is chitin which is found in the cell walls of some of the higher fungi. Considerable doubt exists concerning the validity of many of the studies on the chemical nature of the cell wall, and all that can be concluded with safety is that it is composed of a relatively inert material, whose main function appears to be the provision of mechanical protection to the cell proper. Some protein may be present in the cell wall and exert a functional role, possibly enzymatic in character.

**The Slime Layer.** The cell wall of bacteria appears to be surrounded by a slime layer, which for a given species can vary considerably in thickness with both changes in the nature of the culture medium and hereditarily transmissible variations within a particular strain. When the slime layer is sufficiently thick and firm to have distinct form, it is usually called a *capsule*. The slime layer is of gelatinous or jelly-like consistency and has a very low affinity for dyes, to which it appears to be readily permeable. It is not visible in the dark field or in ordinary preparations, but it can be demonstrated following the application of special positive or negative staining techniques. In some instances its presence has also been demonstrated in electron micrographs of specially treated cells.

Some consider the slime layer to be a modified outer layer of the cell wall arising from the swelling and gelatinization of certain of its constituents. Others claim that the slime layer is a secretory product different in chemical composition from the cell wall. In many species the slime layer or capsule is composed of a polysaccharide gum, typical examples being galactan, levulan, or dextran, which on hydrolysis yield the simple sugars galactose, levulose, or glucose (dextrose), respectively. Cell-free enzyme preparations have been obtained from a number of bacteria, and these preparations are able to synthesize these gums from their constituent sugars. It has been suggested that, at least at times, the slime layer is composed of extracellularly synthesized carbohydrate gums, which are deposited around the cell wall. Mere washing with water frequently serves to extract the slime layer from the cell without damage to the viability of the cell. Hence the slime layer does not appear to be a particularly vital part of the bacterial cell. The composition of the slime layer varies with the species or strain and in some species or in some subtypes consists of nitrogenous material associated with the carbohydrate.

Certain of the higher bacteria form *sheaths*, which are roughly analogous to capsules but which tend to have a firmer structure. In some species of iron bacteria the sheath appears to be primarily a matrix of ferric hydroxide, while in other species the sheath consists of organic matter or of organic matter in which ferric hydroxide is embedded. The sheaths of the filamentous sulfur bacteria appear to be organic in composition.

Capsules may surround each cell in a culture, or when the cells are

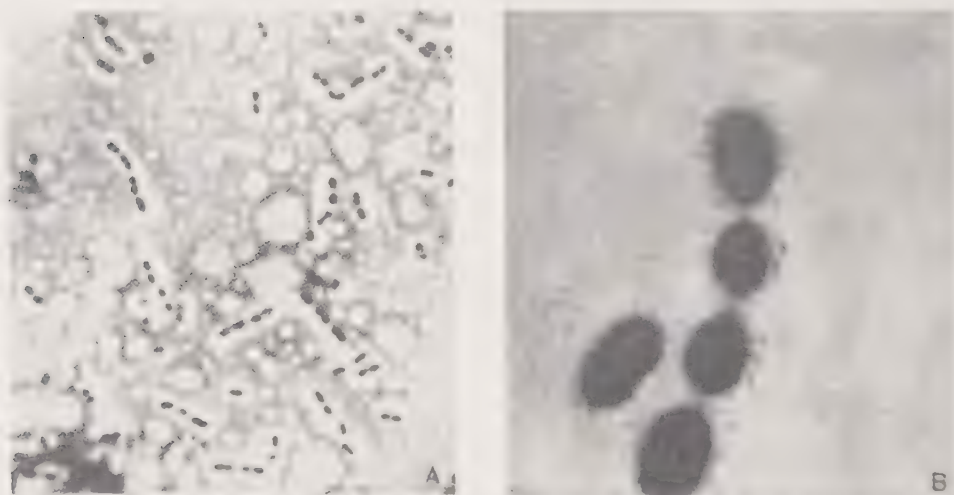


FIG. 3.6 (A) Optical- and (B) electron-microscope photographs of *Bacillus pasteurii* showing capsules. Capsular material aggregated in the electron microscope preparation. (Courtesy of F. W. Schultz.)

characteristically paired or in chains, the capsule may surround all the cells of a group. In some species the capsules of a considerable number of cells may become confluent, giving rise to the formation of a collection of cells known as a *zoogloea*. The zoogloae are frequently encountered in concentrated sugar solutions in sugar refineries, and owing to their high viscosity the flow of sirups through the piping systems is slowed down or stopped entirely.

Capsule formation amongst the pathogenic bacteria has been studied rather extensively because of the fact that the capsular material imparts a high degree of immunological specificity (see Chap. 21) to closely related species or to types of a species, particularly among the streptococci and pneumococci. Furthermore, those disease-producing bacteria which form capsules are among the most virulent (measure of ability to produce disease) pathogens, and capsule formation is considered by some workers as a protective mechanism of the cell against the defensive forces of the host. Variants of a virulent, capsule-producing strain which are weak in or lack the ability to form a capsule are generally much less virulent or entirely avirulent.

The polysaccharides forming the capsules of a given bacterial species differ chemically between the different subspecies or types that comprise a species, while the chemical composition of the cell proper tends to remain relatively constant in the different types. There is only one species of pneumococcus, but this species is composed of more than fifty types, possibly even more than one hundred different types, the types varying only in the nature of their capsular material. It is essential to be able to determine readily the specific type involved in an infection if serum therapy is to be employed. This will be considered in Chap. 21, but it may be mentioned that the capsule of a given type of pneumococcus appears to swell when mixed with an antiserum (serum containing substances [antibodies] which react specifically with cells or cellular material foreign to the animal) developed against this type. No swelling will be observed when the same type of pneumococcus is mixed with normal serum or with antisera against other types of pneumococci. Thus, the *Quellung* reaction, is quite commonly employed for the identification of pneumococcal types and is illustrated in Fig. 3-7. The apparent swelling is the result of deposition of antibodies in and on the capsule, the reaction between the specific compounds (antibodies) in antisera and the capsular material tends to neutralize the ability of the pneumococcus to produce an infection.

Tranveth (1954) has demonstrated morphological differentiation in the capsules of some species of *Bacillus*. The structure of the capsule became evident under the phase microscope, following treatment of the cells with specific antisera (see Chap. 21). The addition of antipolysaccharide





FIG. 3-7. The *Quellung* reaction: capsular material made more evident (appears to be swollen) around cells (b) treated with antiserum.

antiserum resulted in the appearance of dense transverse septa continuous with the cross walls. These septa extended outward to the surface of the capsule. Subsequent treatment of the same cells with antipolypeptide serum demonstrated the presence of polypeptide within the polysaccharide structure. The cell wall, after removal of the capsular material by treatment with the enzyme lysozyme, reacted only with the antipolysaccharide serum. Further treatment with lysozyme dissolved the cell wall polysaccharide, and rounded protoplasts were liberated from the originally rod-shaped cells. This demonstrates the role of the cell wall in determining the shape of the cell.

**The Bacterial Nucleus.** A nucleus can be defined as *a body present in cells capable of further multiplication, which is morphologically distinct from the cytoplasm, which is composed mainly of nucleoprotein, and which alone bears the hereditary characters of the cell.* Nuclei in this sense have been demonstrated in all groups of microorganisms, with the possible exception of the blue-green algae, the bacteria, and highly parasitic forms such as the rickettsiae. The blue-green algae possess a central body which is looked upon as a primitive nucleus—a structure that is not sharply differentiated from the cytoplasm but shows a tendency toward differentiation. It is difficult to demonstrate the presence of a discrete nucleus in bacteria, but there is abundant evidence that a nuclear apparatus is present.

All living cells contain nucleoprotein, and in fact some of the simplest biological entities—the filtrable viruses—appear to consist primarily or entirely of nucleoprotein. The nucleoproteins were originally given this name because they constitute a major portion of the nucleus. They have been shown to consist of nucleic acids in combination with simple proteins. Nucleic acids are complexes of organic bases (cytosine, thymine, uracil, adenine, and guanine) known as purines and pyrimidines, phosphate, and one of the sugars—ribose or deoxyribose. The ribose-containing nucleic acids (RNA) are found primarily in the cytoplasm and cell

membrane while the deoxyribonucleic acids (DNA) are present in the nucleus. There is some evidence that each gene in the nucleus is composed of one nucleic acid molecule.

Bacteria are particularly rich in nucleoproteins, and these compounds have a strong affinity for the ordinary basic dyes employed so commonly for the staining of bacteria. Furthermore a particular type, or combination, appears to be responsible for gram positivity. In many higher cells nuclear structures can be demonstrated directly with the aid of differential stains. RNA-containing materials staining differently from DNA ones. In bacteria there is a marked tendency for the RNA structures to stain so heavily that any differential staining of the nuclear apparatus is masked. Direct differential staining can, however, be accomplished at times. It is much easier to hydrolyze the RNA, either with dilute acids or with a specific enzyme (ribonuclease), so that it no longer masks the staining of DNA. Acid hydrolysis also alters the DNA but sets free aldehyde groups in the deoxyribose (but not in ribose) that react specifically in the Feulgen reaction with basic fuchsin decolorized by sulfite. Basic fuchsin is set free as a result of the reaction and stains the DNA-protein, thus making evident the nuclear material. Controlled hydrolysis, or enzymatic hydrolysis, also alters the cells in such a manner that differential stains such as the Giemsa stain can be employed for differentiation between cytoplasmic and nuclear matter. While the results of such treatments do not yet yield unequivocal support for the existence of nuclei in bacteria, by analogy with similar behaviors noted in higher cells they are highly suggestive. Furthermore, observations of untreated bacteria in phase (Fig. 1-13) or electron (Figs. 3-3 and 3-13) microscopes have revealed the presence in bacteria of bodies or structures of the same size, shape, and location as those noted in hydrolyzed and stained preparations. The various observations support the early contention of a number of workers that bacteria do possess a nuclear apparatus, evidence for which was once less complete than at present. Studies of the division of nuclear material in growing cells under the phase microscope (limited by the characteristics of this instrument), of mutations, and of sexual recombination lend further support to the concept that bacteria possess a nuclear apparatus. A major problem confronting the bacterial cytologist is the nature of this nuclear apparatus.

Discrete Feulgen-staining bodies occurring in regular numbers and exhibiting characteristic division were demonstrated by both Stille and Prokarski in 1937. Studies by Robnow (1944, 1945) and, in particular, evidence presented by him for the existence of paired, chromosome-like nuclear bodies in bacteria contributed greatly to the general acceptance in the United States of the concept that bacteria do possess a nuclear apparatus. Recent studies have been reviewed by Knaysi (1956).

Robinow came to the conclusion that the basic chromatinic element in bacteria is a more or less dumbbell-shaped rodlet which divides lengthwise in a plane usually parallel with the short axes of the bacterium, one "dumbbell" giving rise to two daughter dumbbells. The first division of the chromatinic rodlet is, at times, immediately followed by the constriction of the bacterium to form two cells; at other times, division of the cell does not occur until after two or more divisions of the chromatinic structures. Bisset has associated this behavior with change from smooth to rough morphology (see Chap. 14).

It is not easy to interpret the nature of the minute structures seen in such small organisms as the bacteria, and much work remains to be done before there is general agreement between cytologists. Bisset has concluded that two different types of nuclei occur in bacteria. One type is a spherical or vesicular structure, most commonly found in spores and resting stages of bacteria. A similar structure may be noted during the active phase of growth of certain cocci, *Azotobacter*, and mycobacteria. More commonly the vegetative nucleus is in the form of paired chromosomes or chromosome complexes, the dumbbell-shaped bodies mentioned above. Bisset also postulates that a sexual fusion of this nuclear material can occur at times, followed by elongation of the bacterium, redistribution of chromatinic matter, and fragmentation of the cell to yield a new generation. His schematic illustrations of bacterial nuclei and their behavior are presented in Fig. 3-8, and photomicrographs of stained preparations upon which the sketches are based in part are shown in Fig. 3-9. These and other studies that are not always in agreement suggest that the nu-

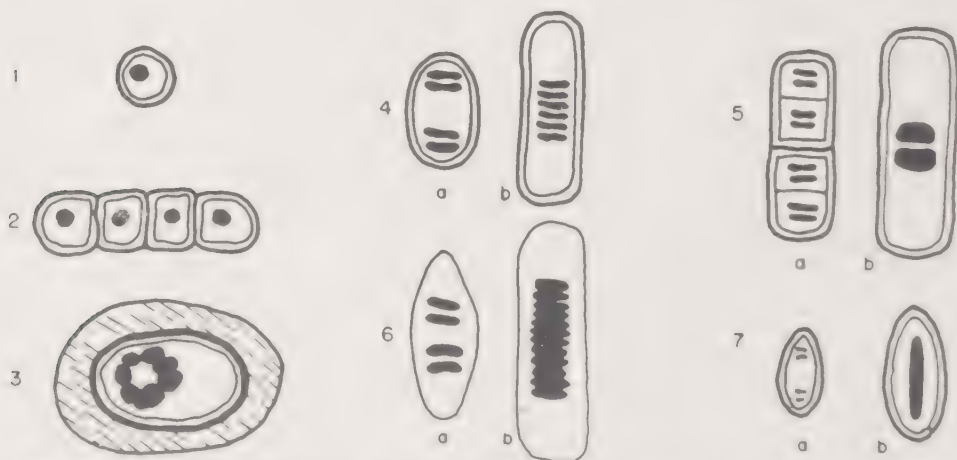


Fig. 3-8. The bacterial nucleus. A discrete nucleus is observed in (1) gram-negative cocci, (2) corynebacteria, and (3) *Azotobacter*; chromatinic structures (a) and fused nuclei (b) in (4) smooth and (5) rough rods, (6) myxobacteria, and (7) short-chained streptococci. Thick line, cell wall; thin line, cell membrane. [After Bisset, *Journal of Hygiene*, 46, 264 (1948).]





FIG. 3-9. Nuclear structures in bacteria. (1-4) Gram-negative coccus: methylene blue-cosin stain except (2), a tannic acid-violet cell-wall stain preparation; (5) methylene blue; (6 and 7) methylene blue-cosin, and (8) basic fuchsin stains of *Azotobacter*; Giemsa stains of corynebacteria (9); of *Shigella dysenteriae* (10); of *Streptococcus* (12); of lactobacilli (13); and of *Chondromyces crassus* (14 and 15) [After Bisset, *Journal of Hygiene*, **46**, 264 (1948).]



clear apparatus may vary with the age of the cells and between species as well. Hence it is necessary to keep an open mind regarding the nature of bacterial nuclei.

Some workers claim to have demonstrated typical mitotic figures in bacteria during different stages of growth (see Knaysi, 1956), but it has not been verified generally. Studies of this nature are hampered by the limits of resolving power of the light microscope, and much work needs to be done before it can be definitely established that the classical picture of mitosis applies to bacteria.

Granted that the transmission of hereditary characters in bacteria is analogous to the process as it occurs in higher cells, it could be assumed that the process takes place through the agency of genes, which must take a fixed position in regard to each other, must synchronize in division, and must be distributed in such a manner that a full complement will be found in each daughter cell. A nucleus, reduced to its simplest components, might then consist of a single string of genes existing as a small granule or as a rod-like body too small to be resolved under the microscope. Genes have been redefined in functional rather than mechanistic terms by Beadle as units able to direct the synthesis of replicas of themselves and serving as models in the formation of nongenetic units of corresponding specificity. They are considered to act as though they are templates for the direction of the synthesis of specific proteins. In this sense genes transmitted from parent to offspring can be considered as sets of master molecules which serve to control the formation of specific counterparts in the following generations, this action of genes being accomplished through the biological catalysts—enzymes—whose configuration, according to one concept, is copied from genes, probably via ribonucleoproteins.

### SPECIAL STRUCTURES OF BACTERIA

**Flagella.** Flagella are the organs of locomotion of the majority of motile bacteria. The filamentous sulfur bacteria and the myxobacteria exhibit gliding movements, the mechanism for which is unknown. All of the curved forms of bacteria and roughly one-half of the rod forms are motile, while very few of the coccoid forms are flagellated. We have considered that the flagella are for the most part too small to be resolved in the light microscope but that they can be observed when stained (see Fig. 2-11) under conditions which lead to marked deposition of dye and of mordanting agent on the flagella. They can be observed most readily in electron micrographs; measurements of typical flagella as observed under the conditions prevailing in the preparations indicating diameters ranging from 0.02 to possibly as high as 0.1  $\mu$  for different species. The lengths of flagella are ordinarily somewhat greater than those of the cells from

which they originate and may be several times the length of the cell. There is disagreement concerning the point of origin of flagella, some believing that they originate in the cell wall or cell membrane while others claim that their origin is in the cytoplasm. The flagella of flagellated protozoa originate in a body known as the blepharoplast, or basal grain, within the cytoplasm, but there is no such structure readily demonstrable within the cytoplasm of bacteria. Electron micrographs of flagellated



FIG. 3-10. Electron micrograph of *Synechococcus* species, indicating intracellular origin of flagella. [From van Iterson, *Biochimica et Biophysica Acta*, **1**, 537 (1947).]

bacteria (Figs. 3-10 and 3-11) indicate that the site of origin of flagella is in the cytoplasm.

**Types of Flagellation.** Examination of many species of bacteria stained to demonstrate flagellation indicates that the type of flagellation is a constant characteristic of a particular species, bearing in mind that a single species may undergo a variation with the loss of ability to form flagella. Bacteria can be divided into five groups by their type of flagellation: the *atrichous*, or nonflagellated, bacteria; the *monotrichous* bacteria possessing a single polar flagellum, the *lophotrichous* bacteria with a tuft of flagella at one end, the *amphitrichous* bacteria with a tuft of flagella on each end, and the *peritrichous* bacteria with flagella distributed over the surface of the cell. There is some doubt at the present time



FIG. 3-11. Electron micrograph of *Serratia marcescens*, suggesting intracellular origin of flagella. (Courtesy of A. L. Hoorink, Institute for Electron Microscopy, Delft, the Netherlands.)

as to the existence of peritrichous flagella, this type of flagellation as observed in stained smears possibly being an artifact produced in the staining procedure. Similarly, the existence of amphitrichous bacteria is doubtful. It has been proposed that flagellated bacteria be divided into two groups: those which possess *terminal* flagella and those which have *lateral* flagella. Little is known concerning the chemical composition of flagella, except that they consist primarily of proteinaceous matter, apparently of a type similar to that found in the elastic, fibrous proteins of mammals. Flagella are not essential to the life of the cell, and variation can occur with the loss of the ability to produce flagella.

**Motion of Flagella.** Flagella can be observed in dark-field preparations of some bacteria and appear to be helicoidal in shape. They form coils which at rest exhibit a high curvature, becoming straighter

and narrower and reforming when the cells are in motion. Flagella propel bacteria, not by a lashing movement similar to that of the cilia of Infusoria, but by rapid periodic contractions which pass through the flagella from one end to the other. This rhythmic contraction, which moves helically through the flagella, must generate considerable force, since some cells can move at a rate as great as  $100\ \mu$  per sec. The average rate of movement of a bacterium such as *Escherichia coli* is approximately  $25\ \mu$  per sec., or about 10 cm. per hr. Direction of movement is apparently controlled by the angle which the flagella make with the cell body; hence the flagella may act both as a propellant and as a rudder.

**Spores.** The family Bacillaceae is characterized as a group of rod-shaped bacteria which form *endospores*. Endospore formation is also observed in a very limited number of cocci and spirilla. These spores are essentially condensations of protoplasmic material (Knaysi, 1952), although they may contain proteins or other substances not found in the vegetative cells in which they were formed, thus suggesting that the formation of endospores is accompanied by the synthesis of new compounds. They are more resistant to injurious agents than are the vegetative cells from which they were derived. Spores may remain dormant for many years, some having germinated after storage for forty or more years in the laboratory. Their resistance is also illustrated by the fact that the time and temperature required for the sterilization of instruments, band- 11  
ages, media, and so on, and for food preservation in the canning industry 12 is directly dependent upon the heat resistance of spores that might be present.

In contrast to the higher fungi, only one spore is formed by each cell, and upon germination each spore gives rise to a single cell. In a number of instances two spores have been reported per cell, but these reports are probably incorrect in most or all cases, since division may have been incomplete and actually but one spore per cell was present. Spores are not reproductive in function in the bacteria in the sense that they do not serve to multiply the species but are essentially *resting forms*, one phase in the life cycle of sporogenous bacteria. 1

When mature, the spore appears as a spherical, oval, or cylindrical body within the cell, and it may be large, thus causing the cell to bulge around the spore, or the spore may be small in comparison with the diameter of the cell, with no change in contour of the cell. The size, form, and position of the spore and the influence of its size on the shape of the cell are fairly characteristic of a given species and are of aid in the identification of species of the Bacillaceae.

**Spore Formation.** Spores tend to appear in the cells as their rate of multiplication begins to decrease, partial depletion of the nutrients in the medium appearing to be one factor influencing spore formation. In gen-





FIG. 3-12. Spore formation in *Bacillus mycoides*. Forespore formation and position of nuclei clearly evident. [From Knaysi and Barker, *Journal of Bacteriology*, **53**, 546 (1947).]

eral, an endospore is formed by a well-nourished cell when conditions for vegetative growth become gradually unsuitable because of either depletion of nutrient material, the development of otherwise unsuitable conditions for growth, and/or unknown "cellular factors." However, the sudden exertion of a harmful condition is not favorable for spore formation, and we do not know the exact elements involved.

The process of spore formation can be followed in either stained or unstained preparations. Three general modes of spore formation have been reported: (1) growth from a single granule within the bacterial cell, (2) an aggregation of granules to form the spore, and (3) condensation of protoplasmic material to form a forespore which upon maturation develops into the endospore. Knaysi favors the latter explanation of spore formation, and on closer examination it becomes apparent that interpretations (1) and (2) are possibly the result of incomplete observation of the phenomenon presented by mode (3). Knaysi (1951) suggests that spore formation takes place in three rather distinct stages. In the first step, granules of lipoprotein, if present, migrate away from the area

within the cell in which spore formation is to occur. A considerable movement of protoplasmic material to this area may be observed, and at the same time a vacuole may develop in the opposite end of the cell. At the same time the nuclei of the cell arrange themselves in two groups at the distal ends of the space to be occupied by the endospore. In the second stage of spore formation, an elliptical envelope of a composition



FIG. 3-13. Nuclear and granular bodies in *Bacillus mycoides*. [From Knaysi and Barker, *Journal of Bacteriology*, **53**, 544 (1947).]

similar to that of the cytoplasmic membrane develops from each group of nuclei, and they merge to give rise to the forespore. Within a few minutes the material within this envelope develops a higher index of refraction of light, i.e., becomes more dense than the surrounding envelope. During the third period, that of maturation, the forespore matures to give rise to the endospore. The remainder of the cell, the sporangium, disintegrates with time, setting the spore free. While the three stages in the development of an endospore discussed above have not been observed in all species, there are suggestions that the same or similar processes are involved in endospore formation amongst the various sporulating bacteria. Knaysi (1952), as a result of studies of living cells of

*Bacillus cereus* under the phase microscope and of stained colonies, described spore formation in this species as follows:

The forespore is initiated by a terminal nucleus of moderate size. Dense material with characteristic staining property is deposited around this nucleus, forming an envelope which grows to the maximum size of the forespore. Prespore inclusions and other nuclei that may be present in the fertile part of the sporangium move, or are pushed, to the sterile part. . . . As far as the forespore nucleus remains visible, it continues to occupy a central position within the forespore. Sometimes the pair of chromosomelike bodies it contains divide forming two pairs. . . . A highly refringent coat is formed within the boundary of the forespore, leaving a peripheral layer which becomes the outer coat of the spore.

Some workers conclude that nuclear fusion precedes spore formation, but the evidence for this is debatable.

The mature spore can be recognized as a refractile, unstained body in the cytoplasm of cells stained by the ordinary or simple stains. However, the spore wall absorbs a small amount of the dye, and free spores would appear as faintly stained bodies in a stained preparation. We have considered that the entire spore can be stained with the aid of penetrating dyes such as carbolfuchsin or malachite green, particularly when heat is employed to intensify the process.

**Properties of Spores.** The bacterial endospore consists of protoplasm of relatively unknown structure but apparently denser or more concentrated than that of the vegetative cell in which the spore was formed. The spore appears to be richer in nucleic acid content than the vegetative cell, to be rich in lipoidal content, and may possess at least one protein ordinarily not present in the vegetative cell. Spectrochemical analysis of a number of spore-bearing species has revealed that endospores are somewhat richer in calcium and manganese and lower in potassium and phosphorus content than the vegetative cells from which they were derived. Protoplasmic material, sporoplasm, appears to be surrounded by two layers, the *intine* and the *exine* coats. It has been suggested that the intine layer, upon germination of the spore, develops into the cell wall of the resulting vegetative cell. The exine layer in some species appears to be absorbed during germination, in other species to be cast off as an empty hulk.

The high resistance of spores to heat and to various chemical agents in comparison with the resistance of the vegetative cells gives the endospores of bacteria a rather unique position in the realm of living things. This marked resistance, as we have seen, was responsible to a considerable extent for the prolongation of the controversy over spontaneous generation. The resistance of the spore to aging, drying, heat, and chemicals has been generally attributed to the possession of a relatively thick, impermeable spore wall and to a low moisture content. Actually we do not

know the true causes. It has been demonstrated that spores lack most or all enzymic activity, one assumption being that the enzymes are combined in some obscure way by their active groups and thus become inactive and heat-resistant. Here, also, the results reported in the literature are somewhat contradictory.

**Germination of Spores.** When spores are transferred to an environment favorable for growth, they germinate, and each viable spore gives rise to a vegetative cell. In some instances at least, the environment need not be a complete culture medium, spores germinating in a sugar solution devoid of nitrogenous matter and utilizing ribonucleic acid in the spores as a source of nitrogen for germination and limited multiplication. The first step in spore germination appears to be a swelling induced by the imbibition of water. At this time a decrease in refraction of light by the spore may become evident. Swelling is most apparent in width and is followed either (1) by cracking of the exine layer (cell wall), polarly or laterally, followed by a casting off of the exine as an empty hull or (2) by a stretching of the cell wall, which in time may be absorbed by the developing vegetative cell. The mode of spore germination may be of some value in the classification of the sporeformers. Knaysi has reported that those species which germinate by shedding their cell wall are more resistant to unfavorable conditions than those in which the exine is wholly or partially absorbed.

**Cell Division.** Multiplication of bacteria takes place primarily by binary fission, and this appears to be entirely asexual in character. It has been reported that the first evidence of division is a constriction of the cytoplasm, the cytoplasmic membrane growing inward until all portions meet in the center of the plane of division. This is followed by a splitting of the membrane and the deposition of cell walls. At times either the formation or the splitting of the membrane is not complete, and connecting links, *plasmodesms* (see Fig. 3-14), can be observed between adjacent cells in a chain. The cells separate when the connecting region of the lateral wall withers away or is split by pressure. When either the breaking down of the lateral wall at the point of division is a slow process or the deposition of the cross wall is a leisurely one, the daughter cells remain attached to each other, giving rise to chains which are characteristic of particular species or genera.



FIG. 3-14 Plasmodesms in streptococci

Many bacteria give rise to at least two different types of colonies, smooth and rough, as will be described in Chap. 14. Smooth colonies,



as the name implies, are relatively smooth and glistening, while rough colonies tend to be wrinkled and dry in appearance. From a study of rough and smooth forms, Bisset came to the conclusion that the constituent bacteria of smooth colonies are typically unicellular, containing two chromatinic bodies. On division, a membranous septum is first formed, the cell then immediately dividing by constriction at this point. The cell walls forming the ends of the new cells are secreted by this membrane which is continuous with the cell membrane, the existing cell wall growing inward as division occurs. In the rough colony the constituent bacteria appear to be multicellular or at least comprised of several cellular units, typically four. Each unit contains a single chromatinic body, these bodies being separated by membranous septa which in time are converted into true septa, by the splitting of which the organism divides. The cell wall appears to be secreted internally in the rough forms, first appearing as a shadow within the membranous septa. Division of rough bacilli occurred at the middle septa, immediately after or during the division of the four chromatinic bodies into eight. Division of rough and smooth variants, as postulated by Bisset, is illustrated in Figs. 3-15 and 3-16. Knaysi (1951), however, concludes that division does not occur by constriction and that the process does not differ between smooth and rough forms. He suggests that chain-forming or rough strains are characterized by the toughness of their walls rather than by mode of division.

The claims that bacteria reproduce by sexual as well as asexual processes rest on (1) observation of cells in contact assumed to be conjugative in character, (2) the formation of bodies strikingly different from the normal form of the species, and (3) recombination between mutants, which can be most readily explained on the basis of a sexual exchange of characters. The evidence for sexual reproduction presented by observations of the nature of types (1) and (2) is not convincing. The studies of Dienes are suggestive, since different strains of *Proteus* growing on the same agar surface give rise in the area of contact with each other to the development of large round bodies. These bodies may undergo a series of changes on nutrient agar and finally give rise to the development of normal bacillary forms of *Proteus*. However no evidence of actual union of cells was observed prior to the formation of the large bodies. A number of attempts to cross closely related species or strains of various bacteria have generally resulted in failure, although recent studies by Tatum and Lederberg indicate that it is possible to produce "crosses."

Tatum and Lederberg employed a number of "biochemical mutants" (see Chap. 14) of *Escherichia coli* in their attempts to obtain crosses with combinations of characters of the original strains. For example, one mutant strain (a) was unable to multiply in a medium devoid of the

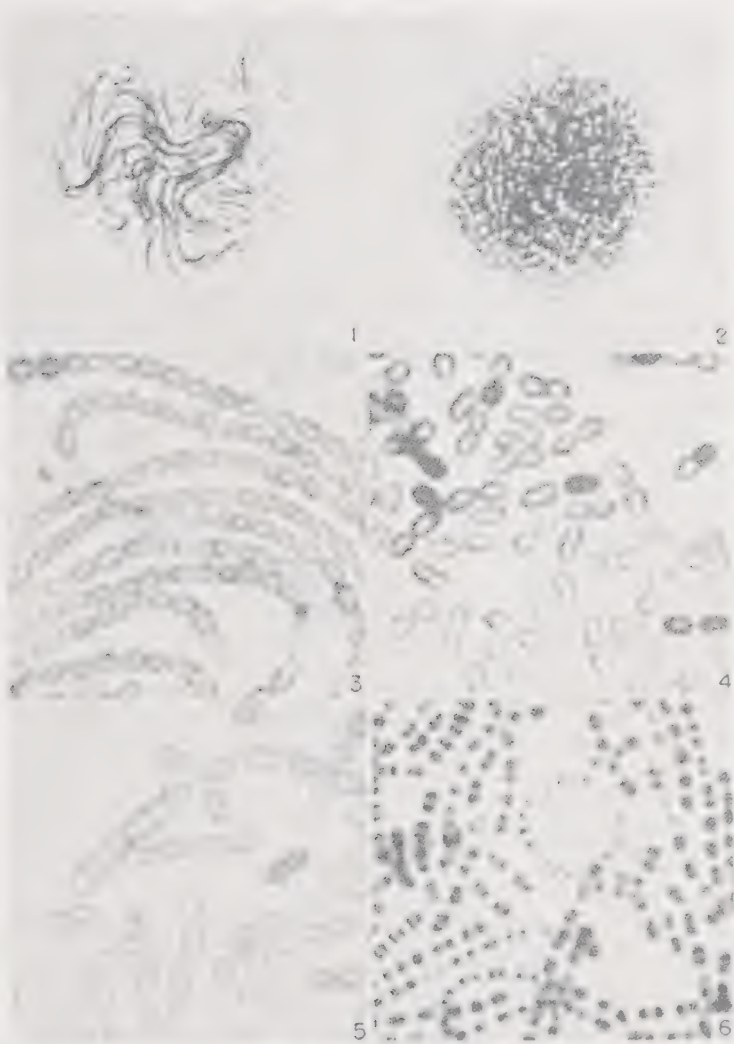


FIG. 3-15 Long-chained (rough, 1, 3, 5) and short-chained (smooth, 2, 4, 6) forms of a *Streptococcus*, illustrating colony form and cell division. [From Besset, *Journal of General Microbiology*, **2**, 129 (1948).]

amino acid methionine and the vitamin biotin. A second mutant (*b*) could develop in the absence of the two substances named above but required the addition of the amino acids proline and threonine to a simple medium before growth took place. When mutants *a* and *b* were inoculated simultaneously into broth and transfers made from the mixed culture to an agar medium lacking all four substances, a few colonies did develop. Evidence of this nature, and analogies with higher forms, suggested the existence of a sexual phase, in which, for example, the genes controlling the synthesis of histidine and methionine in mutant *b* could be transferred to mutant *a* with the production of a cross which could develop in the absence of



A



B



FIG. 3-16. Schematic and photographic illustrations of cell division in smooth (A) and rough (B) variants. [From Bisset, *Journal of General Microbiology*, **2**, 83 (1948).]

lating methionine, proline, and threonine, the latter two genes being originally present in mutant *a*. Recombination of genes controlling the synthesis of a number of compounds essential for growth and of genes controlling the susceptibility of the cells to the lytic activity of bacteriophage (see Chap. 14) have also been noted.

We have considered the general cytology of bacteria and certain characteristics peculiar to these forms of life. Information concerning the nature and structure of bacteria has increased to a considerable extent in recent years, and the healthy interest exhibited at the present time concerning the nature of bacteria suggests that it may be possible in the near future to define and describe the bacteria in more accurate terms. It is desirable to realize that our knowledge is imperfect and incomplete, and we should remember that ideas advanced today may change with the morrow's developments. At this time it might be well to compare what we have considered concerning the nature of bacteria with the general nature of other microorganisms.

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## CHAPTER 4

### THE PROTOZOA AND ALGAE

We have defined microorganisms as forms of life too small to be readily visible with the naked eye, whose distinguishing characteristics become evident only with the aid of a microscope. Microbes differ greatly in size, structure, and mode of life, but they all share an apparently simpler structure and a smaller size than the plants and animals with which we are more familiar.

We have considered in a general way the structure of the smallest microorganisms, the bacteria, but before we proceed to a more detailed study of the bacteria, it is well to review the general characteristics of the various types of microorganisms ordinarily considered in a course in general biology. Water, to which some hay has been added, will abound after a few days in a variety of microorganisms—bacteria, algae, and protozoa. The protozoa and algae can, as a rule, be readily differentiated from the bacteria on the basis of size and the protozoa from the algae by the presence of chlorophyll in the latter group of organisms. There are exceptions to these general statements, and some organisms do exhibit plant-like characteristics in one phase of growth, animal-like in another, thus complicating attempts at classification.

The majority of plant forms possess the photosynthetic pigment chlorophyll, with the aid of which they are able to obtain energy from light for the ultimate conversion or reduction of carbon dioxide to organic matter, probably a carbohydrate, which serves as a source of organic building material for other syntheses carried out by the plant. Carbon dioxide, water, and inorganic salts serve as the initial building materials of the plant cells and are absorbed from their environment. This absorption of relatively simple inorganic compounds from aqueous solution is characteristic of plants and is spoken of as *holophytic*, or plant-like, nutrition. The diet of the higher plants is wholly inorganic in character; considering carbon dioxide and the salts of carbonic acid as inorganic compounds, and such organisms are said to be *autotrophs*, or *autotrophic cells*. This term implies that the plants are self-sufficient in that they do not require an external source of organic foodstuffs as do the animals. The energy for the reduction to cellular material of carbon dioxide by hydrogen from

water is trapped from light by means of the chlorophyll system, so that plants in general can live completely independent of other forms of life as long as light and essential salts, water, and carbon dioxide are available.

All members of the animal kingdom, with a few exceptions, are able to ingest solid masses of food and in the process of digestion convert the raw foodstuff into soluble substances which nourish the cells of which the animal is composed. Both energy and building materials are obtained from the compounds absorbed from the digestive tract. This type of nutrition is spoken of as holozoic, or animal-like. The energy for the synthesis of cellular material and for the various functions of the organism is obtained during the oxidation of the ingested foodstuffs or products of their digestion. Animals are not as independent of other forms of life as the plants, since the animals must be continuously provided with organic matter obtained either from other animals which have fed upon plants or from the plants directly. The few species of animals unable to ingest matter in bulk must obtain all their food and building material by absorption from aqueous solution, in a manner analogous to the plants. Such a mode of animal nutrition is termed *saprozoic*. Actually the majority if not all of the *cells* comprising the animal body are saprozoic in nutrition, the digestive system of the animal providing foodstuff in solution for the individual cells, the term holozoic referring to the over-all picture.

Holophytic nutrition in the respect that foodstuffs must be in solution is one characteristic of the fungi—yeasts, molds, and bacteria—which links them closely to the vegetable kingdom. Yet carbon dioxide does not serve as the sole source of carbon for most species of these fungi, and likewise but few species are able to utilize the energy of light. A few species of bacteria are autotrophic in their nutrition, being able to live and multiply on a wholly inorganic diet, some obtaining their energy from the oxidation of inorganic matter, others from light, and their carbon from carbon dioxide or carbonates. Most species are heterotrophic, i.e., they obtain their carbon and energy from organic matter in solution, a type of nutrition like that of the saprozoic organisms, or cells in general; but since these forms of life are classified in the plant kingdom, their nutrition is said to be *saprophytic*. Fundamentally there is no difference between saprozoic and saprophytic nutrition except that organisms classified as animals are considered in the first term, as plants in the latter.

Saprozoic or saprophytic nutrition is carried to the extreme limit in the *parasitic* plants or animals, which are dependent upon a particular host for their maintenance. As we shall consider later, parasites which produce damage in their host are termed *pathogens*, a term indicating that they produce suffering.

Fungi are generally considered to be members of the plant rather than the animal kingdom because plants and fungi tend to possess a rigid cell

wall, to synthesize and store starch rather than glycogen, to possess a simpler cell structure than animal cells, and to be nonmotile. However there are exceptions to these general statements. While many fungi do possess cell walls composed primarily of cellulose, a substance characteristically of plant origin, yet some do contain chitin or chitin-like material. Many fungi synthesize starch, but many synthesize glycogen, while still others synthesize starch- or glycogen-like substances called, respectively, granulose and iogen. Simplicity of structure is another general characteristic of plant cells, animal cells tending to be somewhat more complex. The fungi do appear to possess a relatively simple structure, but this may be due in part to inability to resolve the structures under the microscope. Also most animals are motile while plants in general are not; some fungi are motile, the majority are not. These different characteristics are not absolute but show only tendencies employed in the differentiation of the smaller forms of life.

It is generally assumed that all life originated from some very primitive unicellular form or forms. As the course of evolution is traced backward from the more complex to the simpler forms, the main branches of both plants and animals appear to come together in the Protozoa. When attempts are made to trace the line of evolution further, distinctions between plants and animals begin to have little meaning, and the simpler forms are so much alike in many respects that each group shows some resemblance to other diverse forms. This difficulty comes to an extreme in the bacteria. Some regard bacteria as products of retrograde evolution—degradation—from somewhat higher forms of life; others consider them to be indicative of the earliest forms of life on the earth.

We have seen that it is difficult to define the bacteria and also to classify them definitely either as plants or as animals. No wonder that years ago Linnaeus assigned them to the order *chaos*! Classification is man-devised and is for convenience in studying the various forms of life. Bacteria may belong in a buffer state between the plant and the animal kingdoms just as the filtrable viruses appear to belong to a buffer state between the animate and the inanimate world. However the bacteria do exhibit many of the characteristics commonly associated with the vegetable kingdom, and therefore they are commonly considered as belonging to that kingdom. There is much we can learn about them, whether they are plants, animals, or neither, and some of the interrelations between the bacteria and other forms of life will become more evident as we proceed with our study of microorganisms.

In the classification and naming of microorganisms the principles employed in systematic botany and zoology are commonly used as a guide. The actual use of these principles as applied to the bacteria will be considered in Chaps. 6 and 15, but before proceeding to a consideration of

the various groups of microorganisms, it might be well to review briefly the general terms which denote the rank of the different taxonomic groups. According to established procedures every individual is assigned to a species, every species to a genus, every genus to a tribe (when necessary—tribes are not always employed), every tribe to a family, every family to an order, every order to a class, every class to a division (the term phylum is frequently employed instead of division), and every division to the plant or animal kingdom. For further classification it is frequently necessary to subdivide the various ranks. This is done by adding the prefix *sub-* to the rank being subdivided. This subclass denotes a group of organisms intermediate between a class and an order, etc. Variations within a species or subspecies may be distinguished by an additional subrank, variety. Classification according to the different ranks can be summarized, together with the suffix indicating the rank, as follows:

	<i>Suffix</i>
KINGDOM	
DIVISION (OR PHYLUM)	
Subdivision	
CLASS	
Subclass	
ORDER	-ales
Suborder	-ineae
FAMILY	-aceae
Subfamily	-oideae
TRIBE	-eae
Subtribe	-inae
GENUS	
Subgenus	
SPECIES	
Subspecies	
Variety	
INDIVIDUAL	

Names of species are, or should preferably be, binary combinations consisting of the name of the genus followed by a single specific epithet. For example, the name *Escherichia coli* means that this bacterium belongs to the genus *Escherichia* and the species *coli*. The generic name in this example was derived from the name of the discoverer of this organism and the species name from the fact that the organism is a common inhabitant of the colon. The generic name should always begin with a capital letter while the species name is not capitalized, although it is permissible but not desirable to capitalize the species name when the species is named after an individual. The names employed are of Latin origin or should be Latinized. An organism *Micrococcus pyogenes* var. *aureus* would be considered as with *Escherichia coli*, to belong to the genus *Micrococcus* and



species *pyogenes* with the additional qualification that it belongs to the variety of *Micrococcus pyogenes* known as *aureus*. The name given (or complete description) after those of the genus and species is the name of the author publishing the name of the organism, e.g., *Thiobacillus thio-parus* Beijerinck signifies that Beijerinck named the organism.

### THE PROTOZOA

In structure the different species of the phylum Protozoa are probably more complex than the other microorganisms included in the study of

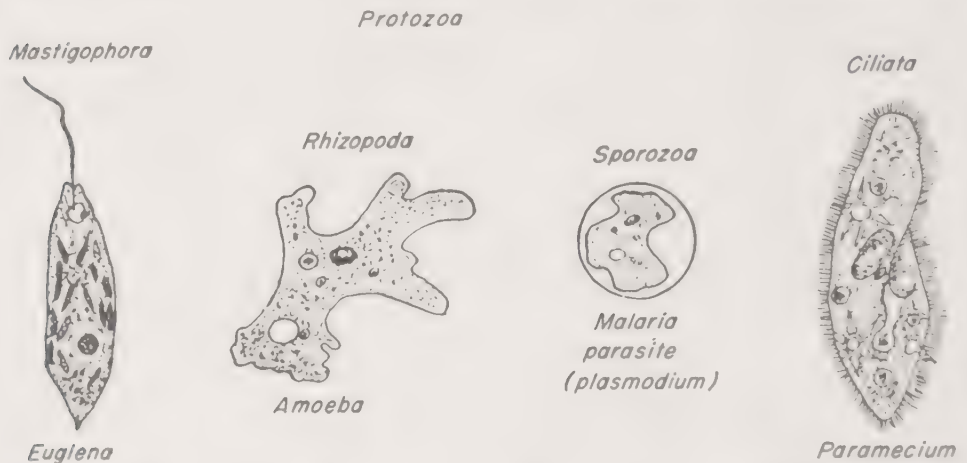


FIG. 4-1. Illustrations of typical protozoa. (From "Biology: The Science of Life," by MacDougall and Hegner, McGraw-Hill Book Company, Inc., New York, 1943.)

microbiology, and except for the fact that a limited number of protozoa are pathogenic, they might not be mentioned in a course in general bacteriology. But certain of these tiny animals do share a number of characteristics with at least one order of the bacteria, and a brief review of their more important characteristics might not be amiss. The protozoa are classified in the division or phylum of the animal kingdom termed **Protozoa**, which is generally divided into five classes as follows:

- Class 1. Sarcodina, or Rhizopoda, motile by means of pseudopodia
- Class 2. Mastigophora, or Flagellata, motile by means of long, whip-like processes, called flagella
- Class 3. Sporozoa, immature forms may be motile by means of pseudopodia; male gamete is flagellate
- Class 4. Ciliata, or Infusoria, motile by means of short, bristle-like processes called cilia
- Class 5. Suctoria, young forms have cilia, while adult forms develop tentacles

**The Sarcodina.** In elementary biology the amoeba are usually considered as the simplest form of animal life. An amoeba consists of a

single continuous mass of protoplasm differentiated into a glossy outer layer, the ectoplasm, and an inner portion, granular and more differentiated, the endoplasm. Within the endoplasm a nucleus and a contractile vacuole can be observed. When an amoeba is at rest, it may be round in form, but the living cell is usually in motion. Since this motion is produced by a sort of flowing of the protoplasm into portions of the cell which are projected as finger-like processes called *pseudopodia*, the amoebae are constantly changing form. This indicates that there is no rigid cell wall.

Amoeba in general feed upon other microorganisms, which they are able to engulf. When a mass of food is encountered by an amoeba, one or more pseudopodia wrap themselves around it, flow together, and take the food into the cell. Here it is contained in a droplet of water, and into this vacuole digestive juices are secreted. Hence the amoeba provides itself with a stomach as banquet conditions demand. The products of digestion are absorbed and waste products excreted, a typical example of holozoic nutrition. The contractile vacuole appears to function primarily in maintaining the water balance between the cell and its environment, and its function may be similar to that of the urinary apparatus of higher animals. We should bear in mind that in the amoeba or in higher animals the nutrition of *the bulk of the cell* in unicellular forms or of the individual cells in higher forms is *saprozoic*, the digestive apparatus of animals converting the foodstuff into soluble matter utilizable by the rest of the organism. Hence, ultimately the nutrition of the individual cell, plant or animal, is essentially similar in that nutrient soluble material is essential. It is also becoming apparent from studies in comparative biochemistry that the chemical reactions involved in maintenance and growth of all forms of life are also very similar in their essential characteristics. There appears to be a greater unity throughout nature than was apparent in earlier years.

The reproduction of an amoeba is relatively simple, the nucleus dividing into two parts, following which the cell itself separates into two parts, each with a nucleus. In the amoeba we have multiplication by binary fission, and it is an asexual process. After a number of generations an amoeba may come to rest in a rounded form and develop a cell wall, resulting in a form known as a *cyst*, which is more resistant to environmental factors. Encystment is more common in the parasitic species than in the free-living forms. Most species are free-living, and many may be found in hay infusion. A few amoebae are parasitic, and one in particular, *Endamoeba histolytica*, is pathogenic for man, being the causative agent of amoebic dysentery.

**The Mastigophora.** Many different kinds of protozoa, both on the basis of structure and of mode of life, are classified as Mastigophora since

they all possess the same type of organs of locomotion, flagella. The flagella are long whip-like processes originating in a blepharoplast (or basal granule) within the cytoplasm of the cell. They generally arise from the front or anterior end of the cell and by an active vibration or by lashing about serve to pull the organism forward. The cell structure of the flagellated protozoa tends to be more complex than that of the amoeba, and many of the flagellates possess distinct structures or specialized areas which serve for the ingestion of food particles or the excretion of waste materials.

The majority of the flagellates are definitely holozoic in their mode of nutrition, but some forms possess chlorophyll in bodies known as *chloroplasts*. Those definitely holozoic in nutrition are frequently classified as *Zoomastigina*, those possessing chlorophyll and holophytic in nutrition as *Phytomastigina*. Little difference in structure can be noted between the two subclasses, and classification of the chlorophyll-free forms as animals and of the chlorophyll-containing forms as plants is hardly worth while. Also certain species are able to engulf solid food particles while others employ only foodstuff in solution.

Reproduction is by binary fission in most species, and in contrast to the rod-shaped bacteria, division occurs in the plane of the long axis, i.e., is longitudinal rather than transverse. Following cell division the two individual cells become completely separated. However, in some species the cells tend to remain together, either as free-swimming masses of cells or as masses attached to submerged material. These masses of cells, or colonies, are held together by a gelatinous secretion, and they are considered to be aggregates of individual cells rather than single multicellular forms, because there is no apparent differentiation of the mass into collections of cells or tissues possessing different functions or structures. However, in a few species there appears to be an elementary differentiation within the colony, since one or more of the cells may undergo multiple rather than binary division, giving rise to more than two cells. Other cells in the same colony may become greatly enlarged and serve as ova to which the small, actively motile cells formed by multiple division migrate and with which they then fuse. After this fertilization of the female cell by the male cells, the large cell may develop a new colony. Here, then, we may have asexual or sexual reproduction within the same species and a degree of differentiation within the colony into vegetative and reproductive cells. Encystment is also noted in the Mastigophora. Some workers suggest that the flagellates may have been primitive forms from which all higher forms of life could have evolved, and even possibly simpler forms by retrograde evolution. In the flagellates differentiation between plants and animals and between unicellular and multicellular forms becomes difficult.



Flagellated protozoa are widely distributed in nature and may be found in hay infusions in a variety of forms. Certain photosynthetic species are of considerable importance in the oceans, as they serve as a source of food for higher animals including fishes. Other forms occur in fresh water and in the soil while a few species are parasitic and pathogenic, *Trypanosoma rhodesiense* and related species producing sleeping sickness and *Leishmania donovani* and similar forms being responsible for certain ulcerative conditions. Many of the pathogenic forms undergo several stages in their development, some of the stages in their vertebrate host, the others in an invertebrate host, and hence the infections are frequently insect-borne.

**The Sporozoa.** The protozoa included in this class are parasitic forms which possess a somewhat simpler structure than the majority of the microanimals, simplification of structure probably resulting from their dependence upon a host for their existence. As regards nutritional characteristics these forms are primarily or entirely saprozoic, and hence there is no need for a complex food-ingestion apparatus or digestive vacuoles. Certain species show amoeboid movement during a period of their life cycle, but in general they do not possess specialized organs of locomotion, with the exception that male gametes may be flagellated.

Two general modes of multiplication, sexual and asexual, are involved during the life cycle of Sporozoa. A cell may undergo segmentation, *schizogony*, with the formation of a number of offspring, and this asexual mode of division permits rapid multiplication of the species to occur within the host. Cells multiplying by segmentation are called *trophozoites*. In some manner certain of the cells become differentiated from the majority of the trophozoites, giving rise to *gametes*, which fuse, and the fusion body divides with the formation of a different form of the species, the *sporozoite*. This phase of multiplication in the life of the parasite is termed *sporogony* and is sexual in character. Certain species of Sporozoa undergo sexual reproduction in one host, asexual reproduction in a different host species, while other species may multiply by both methods in the same host. The malarial parasites multiply by schizogony in man, by sporogony in the mosquito. A number of days are required for the completion of the life cycle in the mosquito before the parasite becomes infective for man. Sporozoites produced in the mosquito are transmitted in its saliva to man when an individual is bitten by the infective insect. In man the sporozoites undergo change with the formation of trophozoites, and these multiply by schizogony in the human host. The malarial parasite is not transmissible directly from one individual to another except in rare instances when the blood of an infected individual is transfused into a noninfected person. Other species of Sporozoa may complete their life cycle in one host, the sporozoites being discharged from



the host encased in a protective covering which gives them a spore- or cyst-like appearance or structure. These more resistant or spore-like forms remain dormant until they enter another member of the host species, generally via food or water in human parasitic forms, where they transform into trophozoites.

**The Ciliata.** In this class there is a marked tendency for the cell to be differentiated into a number of specialized parts, *organelles*, analogous to organs in multicellular forms. The Ciliata are characterized by the possession of cilia, relatively short bristle-like processes distributed over the surface of the cell, whose function is to propel the cell. Propulsion is accomplished by a backward lashing movement of the cilia somewhat analogous to the propulsion of a boat by means of oars, as contrasted with the more propeller-like traction of the flagellates. Each cilium arises from an end plate directly under the surface of the cell. While the cells of a particular species generally maintain a definite, characteristic shape, they are able to undergo considerable deformation, and hence the cell membrane appears to be somewhat more elastic than that of the bacterial cell. Multiplication is primarily by fission although sexual reproduction may also be observed.

Typical members of the Ciliata are found in the genus *Paramecium*, which is familiar to all students of biology. *Paramecia* ordinarily are found in hay infusion, along with other protozoa. The *oral groove* running diagonally across one side of the cell of a paramecium serves as a food-collecting structure, and it leads to a short, funnel-like opening into the cell known as the *gullet*. Water droplets bearing food particles, generally organisms considerably smaller than the paramecium, enter the cell through the gullet with the formation of a vacuole within the cell. These vacuoles follow a definite pathway through the cell and during their migration serve as organs of digestion, hydrolytic enzymes in particular being taken up from the cell proper and products of digestion within the vacuole being absorbed by the protoplasm of the paramecium. Here again we see that the cell as a unit is holozoic but that the nutrition of much of the cell is saprozoic. Undigested material is excreted from the cell when the vacuole reaches the *anal opening*, the end of its path of migration through the cell. Hence the paramecia have "self-forming, migratory organs of digestion" and a definite structure for the elimination of undigested material. Other structures, contractile vacuoles, are involved in the maintenance of the water balance between the cell and its environment and possibly also in the elimination of waste products.

*Paramecia* are also characterized by the possession of two nuclei, a larger *macronucleus*, which is believed to control the metabolic activities of the cell, and a smaller *miconucleus*, which apparently bears the hereditary characters of the species. When a paramecium divides, both nuclei

undergo division preceding division of the cell, which is accomplished by transverse fission, i.e., at right angles to the long axis, instead of along this axis as in the flagellates. Division of the macronucleus is accomplished by elongation of this body followed by a constriction which separates the nucleus into two halves. This relatively simple mode of nuclear division is said to be *amitotic* in contrast to the more complex, ordinary mode of nuclear division by mitosis. Division of the micronucleus, as is also true for the nuclei of other protozoa, is mitotic in character. Multiplication is for the most part asexual, but after a number of generations two cells may conjugate, a specialized process in which the two cells are united by a narrow tubular bridge. Rather complex changes occur in the micronuclei of the conjugated cells, while the macronucleus does not appear to be involved in the process and, in fact, disappears. Ultimately the two cells are cross-fertilized, separate, and the micronucleus in each is involved in a series of divisions resulting in the formation of new macronuclei and micronuclei. Following a series of cell divisions, each cell ultimately contains but one micronucleus and one macronucleus. This process, analogous to changes observed in sexual reproduction in higher forms of life, does not appear to be absolutely essential for the propagation of the species since many species may be carried through numerous generations without any evidence of conjugation.

The Ciliata are structurally highly complex unicellular forms when compared with the amoeba and bacteria. The majority of the species are free-living forms and are widely distributed in nature although there are a few species which are parasitic. *Balantidium coli*, an intestinal parasite of hogs, is the only ciliate which appears to be pathogenic for man.

**The Suctorina.** These organisms, often included under the Ciliata, are the most complex members of the Protozoa. They exhibit two distinct growth phases, the young cells being free-living, ciliated forms which later become attached to a solid object, lose their cilia, and develop tentacles. The tentacles are long tubular processes with a swollen extremity in which food particles, generally ciliates, become attached. The food-stuff is sucked through the tentacle into the body of the cell. Reproduction is mainly by budding in a manner somewhat analogous to yeast. The buds are pinched off the parent cell, develop cilia, and enter the multilevel phase of the growth cycle. The Suctorina are for the most part free-living forms.

It is apparent that the phylum Protozoa is composed of widely diverse microorganisms having in common the unicellular state and a predominance of so-called animal characteristics over plant characteristics. Some of these organisms approach in complexity the multicellular state, while others are relatively simple forms of life. The majority are free-living

forms whose natural habitat is water; a number of species are found in the soil, and a still more limited number are parasitic in or upon plants and animals. The more common forms pathogenic for man are listed in the key below, together with a greatly abridged general description of the organism and mention of the infection produced.

#### KEY TO THE MORE IMPORTANT PROTOZOA PATHOGENIC FOR MAN

- I. Motile by means of pseudopodia. *Sarcodina*
  1. *Endamoeba*, cysts contain no more than four nuclei
    - histolytica* . . . . . Amoebic dysentery. May also invade body and infect liver, lungs, and other organs
- II. Motile by means of flagella. *Mastigophora*
  - A. Elongated, spindle-shaped, flexible cells
    1. *Trypanosoma*, generally four developmental stages
      - gambiense, rhodesiense* . . . . . African sleeping sickness
      - cruzei* . . . . . Charga's disease in Brazil, an infection similar to above
      - equiperdum* . . . . . Equine syphilis (dourine)
    2. *Leishmania*, similar to *Trypanosoma*, two developmental stages
      - donovani* . . . . . Kala azar (black fever)
      - tropica* . . . . . Oriental sore
      - brazilienses* . . . . . Similar to Oriental sore, but infection tends to spread to greater extent
- III. Motile by means of cilia. *Ciliata*
  - A. Parasitic in intestinal tract
    1. *Balantidium*, large ovoid cell commonly parasitic in swine
      - coli* . . . . . May cause a mild dysentery in man
- IV. Strict parasites with complex life cycle, asexual reproduction by multiple fission (schizogony), alternating with sexual reproduction (sporogony) to give infectious sporozoites, immature stages motile by pseudopodia, male gametes flagellated. *Sporozoa*
  1. *Plasmodium*, part of life cycle in man, part in mosquito
    - malariae, vivax, falciparum, ovale* . . . Malaria

#### THE ALGAE

Bacteria, yeast, molds, and the higher fungi are classified as "simple plant forms which do not possess chlorophyll." They were (see Chap. 6) included together with the algae, which do contain chlorophyll, in the

phylum or division *Thallophyta* of the plant kingdom. The thallophytes are plant forms which show no differentiation into roots, stems, or leaves in contrast with the moss-like plants, *Bryophyta*; the fern-like plants, *Pteridophyta*; and the seed-bearing plants, *Spermatophyta*. The *Thallophyta* is the only group of plants generally included in the study of microbiology.

The algae comprise a subphylum of the *Thallophyta* termed the *Algae*, while the various rusts, smuts, mushrooms and related forms, yeasts, molds, and bacteria form the second subphylum, the *Fungi*. The algae are generally divided into five classes:

Class 1. *Diatomeaceae* (*Chrysophyceae*), the diatoms or yellow algae, sometimes classified in Class 4

Class 2. *Cyanophyceae*, the blue-green algae

Class 3. *Chlorophyceae*, the green algae

Class 4. *Phaeophyceae*, the brown algae

Class 5. *Rhodophyceae*, the red algae

The fungi may be further subdivided into the *Eumycetes*, or true fungi (yeasts, molds, and higher forms), and the *Pseudomycetes*, or false fungi (bacteria and slime molds). Their classification and general morphology will be considered after the algae and also in subsequent chapters. The fungi appear to be of most importance to man as they are directly responsible for the maintenance of soil fertility, the disposal of waste matter, the spoilage of food and in other instances its preservation or improvement, and finally as the cause of various diseases of man or other animals or of plants. But the algae are also of great value to us as individuals, for while the grasses and grains serve as food for our meat-providing animals, the algae are as important as forage plants for both fresh- and salt-water fish. In addition they can cause trouble by their presence in water supplies to which they impart disagreeable odors and tastes, and by enriching the organic content of the water, enhance the possibility of survival of pathogenic organisms which may gain entrance to the water supply.

We have considered that certain of the flagellated protozoa are plant-like, or holophytic, in their nutrition; in particular, those species which possess chlorophyll are very closely related in their nutritional characteristics to the photo-synthetic plants. These organisms may be considered as transition forms between the plant and animal kingdoms.

**The Chlorophyceae.** Some of the simpler types of the chlorophyll-possessing flagellates exhibit two growth phases. In the phase (palmella) most suggestive of plant life, the flagellates lose their flagella and surround themselves with a gelatinous secretion. This process appears to resemble encystment, but actually the cells are still growing and dividing and in time give rise to a colony of nonmotile cells enclosed within a jelly-like sheath. This to the botanist, represents a plant, but from it flagellated



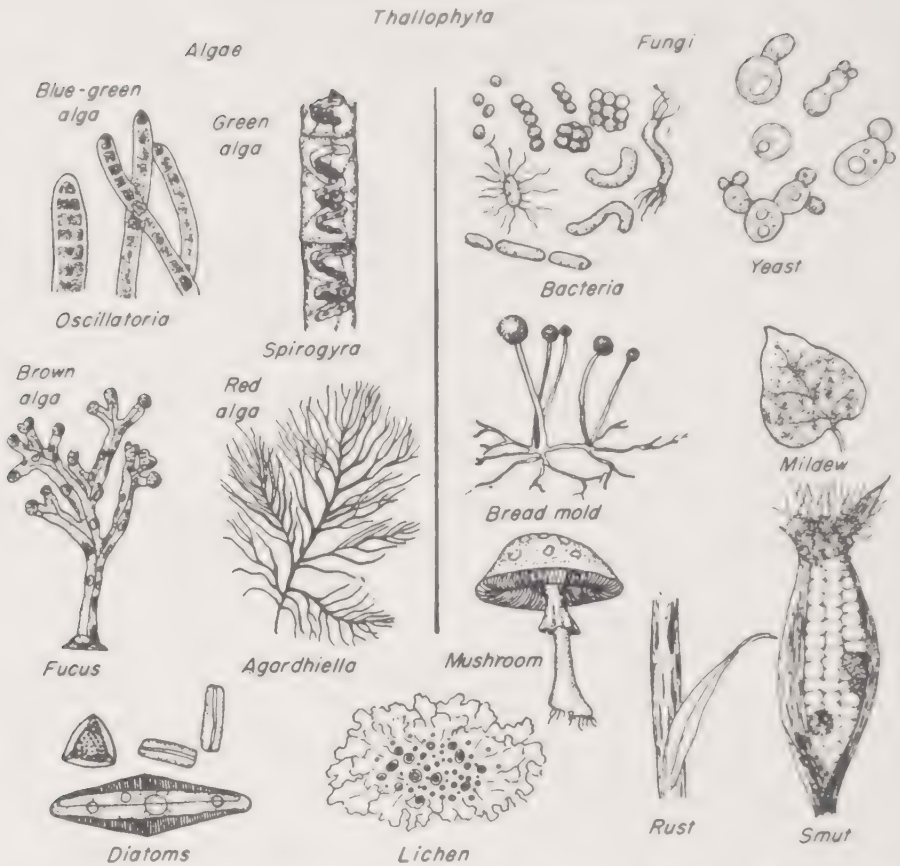


FIG. 4-2. The Thallophyta. (From "Biology: The Science of Life," by MacDougal and Hegner, McGraw-Hill Book Company, Inc., New York, 1943.)

cells may break away to serve as reproductive bodies for maintaining and scattering the species.

There is a wide variety of forms included in the green algae, and these are classified primarily according to the character of the reproductive cells, whether the forms are unicellular or multicellular, and by the form and arrangement of the chloroplasts within the cells. Small bodies known as *pyrenoids* may be observed within the chloroplasts, and these appear to be composed of a central proteinaceous granule surrounded by starch, and their function appears to be that of a center of starch synthesis. Synthesis of starch and the possession of cellulose walls link many of the green flagellates closely with the plant kingdom. Also the filamentous types of growth of some of these green algae, with or without the formation of cross walls in the individual threads or filaments, bear resemblance to those of the filamentous fungi.

**The Rhodophyceae and Phaeophyceae.** These algae for the most part cannot be considered as microorganisms since many of them form micro-

scoptic plants, some reaching a length of several hundred feet; yet they do not possess well-developed roots, stems, or leaves. They may have originated from the green algae but differ from them in the possession of various pigments in addition to chlorophyll, in their larger size, and generally in more complicated modes of sexual reproduction. Some of the multicellular forms are actually a mass of more or less independent forms clinging together, and a single cell by simple fission can give rise to the establishment of a new plant. Certain of the red algae are of fundamental importance to the bacteriologist as they serve as the source of agar-agar, the jelly-like base of most of our solid media.

**The Diatomaceae.** The Diatomaceae—or Chrysophyceae, or Bacillariaceae, as they are also known—differ from the green algae in that they possess the yellow pigment, xanthophyll, in addition to chlorophyll in their chloroplasts. This gives rise to a yellowish appearance on the part of a group of cells. They also differ in that they appear to synthesize and store in oil as a reserve food product in place of starch. Most of the yellow algae are unicellular, and a few species are animal-like in their characteristics and hence claimed by both botanists and zoologists. One group of yellow algae, the diatoms, is characterized by the formation of siliceous shells. Upon death of the individual cell these shells settle to the bottom of the body of water, and enormous deposits of this material—diatomaceous earth—have been uncovered at various places. Diatomaceous earth is used to a considerable extent as a polishing powder and is also a base of certain bacteria-retaining filters employed in the laboratory, particularly for the removal of bacteria from culture media and in the study of filtrable viruses. The diatoms may float free in the water or may grow attached to other material by means of a gelatinous stalk formed by the cells. These algae, directly or indirectly, serve as a highly important food for fish in the oceans.

**The Cyanophyceae.** The blue-green algae, sometimes called Schizophytes, are the simplest forms of green plant life and form a class rather sharply differentiated from the other algae, at the same time exhibiting many characteristics of the bacteria. The blue-green algae are for the most part unicellular forms and are usually bound together to form colonies by a common gelatinous covering, or are connected to form filaments frequently enclosed within a sheath. They have become adapted to a wide variety of habitats, and while they are essentially aquatic plants, yet they may lead a semiterrestrial existence in damp places. A few, like the thermophilic bacteria, have become adapted to growth at elevated temperatures (50 to 60°C. or higher) which are inimical to the growth or even existence of the common plants and animals. While most species are photosynthetic, a few species are saprophytic in their nutrition, and certain of these forms are semiparasitic upon higher aquatic

plants or animals. These algae grow free or attached to rocks or aquatic plants, the free or floating forms comprising the bulk of the vegetable plankton of fresh water. Under favorable conditions they multiply rapidly, giving rise to a green scum on small bodies of water.

The blue-green algae differ from the green algae in that their chlorophyll is not contained in chloroplasts but instead is diffused, along with other pigments, throughout the protoplasm. The bluish color of many species is due to the presence of the pigment phycocyanin, but other pigments can also be present and give rise to brown, olive, or yellow forms. Various granules of reserve food materials are present within the cells of many of the species. One widely distributed type of granule, volutin, composed of nucleic acid, is also found in some species of green algae, in diatoms and protozoa, and particularly in yeasts and in certain bacteria. Carbohydrate is stored in the form of glycogen rather than starch, a characteristic of fungi or animals rather than plants, and oil droplets are also commonly encountered. When these algae multiply to a considerable extent in water supplies, they cause disagreeable odors, primarily owing to their oil content. They can be controlled by the addition of minute amounts of copper sulfate to the water supply.

Reproduction of the blue-green algae, like that of the bacteria, appears to be primarily asexual and is almost exclusively by simple cell fission. In some species, certain cells pass into a resting stage characterized by an enlargement of the cell with the development of a heavy cell wall and an accumulation of reserve food material. As with the spores of bacteria, these spore-like structures do serve not as a means of reproduction but rather as a resting stage. There is no distinct nucleus in the blue-green algae, in contrast to the more readily apparent nuclei observed in the other classes of the algae. There is, however, a rather poorly differentiated central body which stains more deeply than the bulk of the cell, and this is generally regarded as a rudimentary or *incipient nucleus*.

The blue-green algae appear to have more in common with the bacteria, other than the possession of chlorophyll, than they do with the other algae, although some forms of the Rhodophyceae are rather closely related to certain species of the Cyanophyceae. Neither of these classes of algae contain any species possessing flagellated forms at any stage of their existence, while flagellated forms can be found occasionally in species of the other three classes. The Cyanophyceae have been placed with the bacteria in a separate class in a proposed classification (see Chap. 6).

## CHAPTER 5

### THE TRUE FUNGI

We have just considered that the thallophytes include the fungi, in addition to the algae. The subphylum Fungi has been divided into six classes as follows (for proposed new classification see Chap. 6):

Class 1. Phycomycetes, alga-like fungi

Class 2. Ascomycetes, the sac fungi

Class 3. Basidiomycetes, the club fungi

Class 4. Fungi Imperfecti (Hyphomycetes), a heterogenous group

Class 5. Myxomycetes, the slide molds

Class 6. Schizomycetes, the fission fungi or bacteria

In the above classification the first four classes represent the true fungi or Eumycetes. The Myxomycetes consist of a borderline group of organisms, protozoan in character during the vegetative stage of their existence (classified as Mycetozoa by the zoologists) but reproducing by spores borne on stalks in a manner analogous to that observed with many fungi. They comprise an interesting group of organisms and afford another opportunity for illustrating similarities between the simpler plant and animal forms. However these organisms were mentioned under the microanimals and will not be considered further.

The Eumycetes are either unicellular or multicellular forms, although single cells in the form of spores do occur in some stage of the life history of the multicellular forms. These fungi tend to produce an intertwining, thread-like mass of vegetation called a *mycelium*, although in a few forms this mycelial type of growth is rarely if ever encountered. This is particularly true with the yeasts, which might be regarded as retrograde fungi in that they appear to have lost the ability possessed by their ancestors of producing a mycelial type of growth.

The algae are considered as primarily holophytic, photosynthetic (energy obtained from light) forms of plant life, while most fungi are devoid of chlorophyll and lead a saprophytic, chemosynthetic (energy obtained from oxidations) type of existence. In contrast to the algae, which are primarily aquatic forms, the fungi for the most part are terrestrial in habitat. While the majority are saprophytic in nutrition, a number of species are parasitic on plants and to a lesser extent on animals.



either as simple parasites or as pathogenic agents. Many of the fungi are able to attack solid foodstuffs but, being unable to ingest them, must therefore secrete digestive enzymes which hydrolyze the food particles into simpler molecules absorbable by the cell. The amoeba creates its own stomach as need arises; the fungus borrows its own environment as a place of partial digestion. Some of these excreted enzymes are employed by man, one enzyme, diastase, being incorporated into certain types of fondant where, under a chocolate cover, it breaks down higher sugars into a semiliquid form with the production of a delectable confection.

The smaller, true fungi are popularly divided into two groups, the yeasts and the molds, but neither of these terms has real scientific significance. There is a tendency to consider the molds as multicellular microscopic forms and yeasts as unicellular forms, but this is not entirely true and both yeasts and molds are classified together in at least two classes of the Fungi, some species being multicellular under certain conditions, unicellular in others. For convenience only, we shall first consider those organisms commonly considered as molds and then proceed to a general discussion of the yeasts.

### THE MULTICELLULAR FUNGI

**General Structure.** The multicellular Fungi are commonly characterized by a nucleated mass of thread-like or filamentous structures, the *hyphae* (singular, *hypha*), which intertwine to form the *mycelium*, or nonreproductive part of the plant. In many instances the mycelium can be differentiated into two main parts: (1) the vegetative hyphae which burrow into the material upon which the fungus is growing and whose essential function appears to be the procurement of nutrient material, and (2) the reproductive hyphae which generally extend into the air and upon which the asexual spores are borne.

In some fungi the hyphae are continuous threads with no apparent cross walls, nuclei being scattered throughout the length of the hyphae. Such forms are said to be *nonseptate*, or *coenocytic*. The cytoplasm may appear homogeneous in the young hyphae, but vacuoles and granules of various reserve food materials, possibly glycogen, volutin, or fat droplets, tend to appear in the older cells. In other fungi transverse walls, or *septa*, form across the hyphae, dividing the threads into a number of individual cells which contain one or more nuclei. In some species the cells are multinucleate in one stage of development, uninucleate in another.

**Asexual Spore Formation.** Both sexual and asexual reproduction occur in the true fungi, minute reproductive bodies known as *spores* being

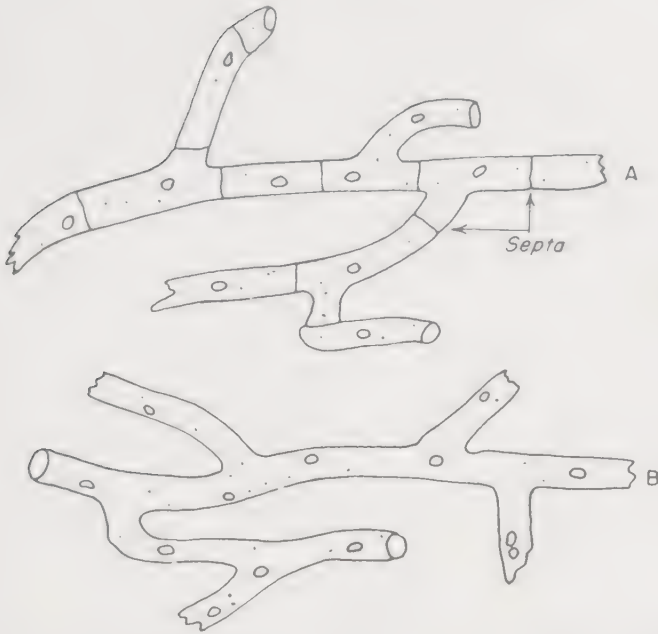


FIG. 5-1. Schematic illustration of septate and nonseptate hyphae.

formed by a variety of methods. The simplest mode of asexual reproduction appears to be by fragmentation of, or by budding from, the hyphae. Septate hyphae in the vegetative mycelium may be constricted into a number of small individual cells, which separate at the cross walls. The cells, called *aridia*, set free in this manner are regarded by some workers as primarily growth forms rather than as spores, since they can continue to grow and to multiply themselves or to produce new mycelia on being set free from the parent plant. In general they do not have so high a resistance to inimical agents as the true spores. Others consider these cells as spores, and they are frequently classified as *arthrospores*. *Oidia* may be cylindrical, oval, or globular in shape, depending upon the species of

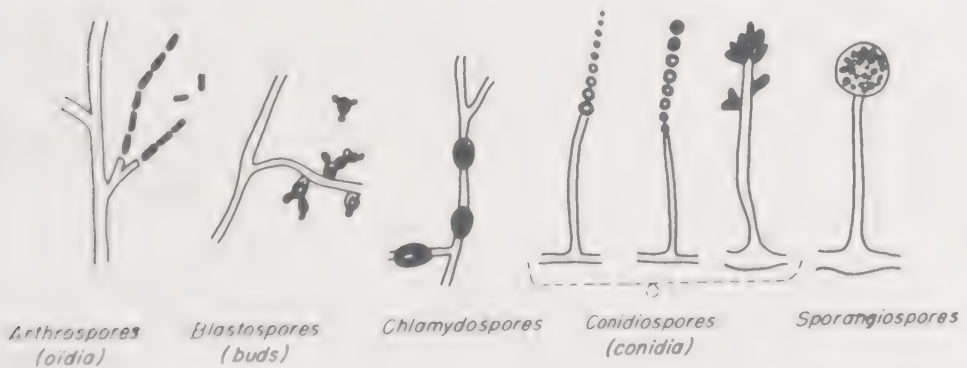


FIG. 5-2. Schematic illustration of asexual spore formation.

fungus producing them. In still other species, somewhat similar spores or growth forms are produced from the hyphae by means of a budding process. Bulges form on the mycelium; a bulge enlarges and constricts at its base with the formation of a bud. The term *blastospore* is at times employed for the cells or spores produced by the budding process. The bud is set free when constriction is complete, and the bud cell so formed may continue to grow and multiply with the formation of a new plant.

In many species budding continues for a period of time, one or more daughter buds forming on the original bud and the daughter buds in turn continuing the process. Oidia, or buds, are formed by a considerable number of species in the different classes of Fungi, the degree of fragmentation or of budding varying with the nature of the species and of the environment. For example, the causative agent of blastomycosis, an infection of the skin and also of the lungs, in which it produces a disease similar to tuberculosis, appears as a unicellular yeast-like form which reproduces by budding within the host or in pus but grows as a multicellular fungus with septate mycelium on artificial culture media. Certain species grow as unicellular forms under conditions of partial anaerobiosis and as filamentous forms under highly aerobic conditions, while in other species the reverse may hold true.



FIG. 5-3. Photomicrograph of a sporangium of *Mucor mucedo*.

True asexual reproductive bodies, *spores*, appear to be richer in reserve food materials, to possess thicker walls, and to be more resistant to inimical agents than the types of spores or growth forms described above. These spores are of two general types: the common spores, which are formed in abundance (often hundreds or thousands per plant) at the tips of fertile or reproductive hyphae, and the less numerous, ensheathed spores, *chlamydospores*, which usually develop within the mycelium. A cell becomes enriched in reserve food material, and its wall markedly thickens to give rise to a chlamydospore, which appears to be a resting stage in the life of the fungus and to be analogous to the cysts of protozoa or to the resting spores of the blue-green algae.

The more common asexual spores, when produced within the cell, are called *endogenous spores*, while those produced externally at the tips of reproductive hyphae are called *exogenous spores*. Endogenous spores are formed when a hypha designated as a *sporangiophore* becomes differentiated from the rest of the mycelium to form a spore case, or *sporangium*, within which the multinucleated mass of protoplasm forms a considerable number of *sporangiospores*. Generally the sporangiophore is enlarged at the tip to form the *columella*, which lies within the sporangium and

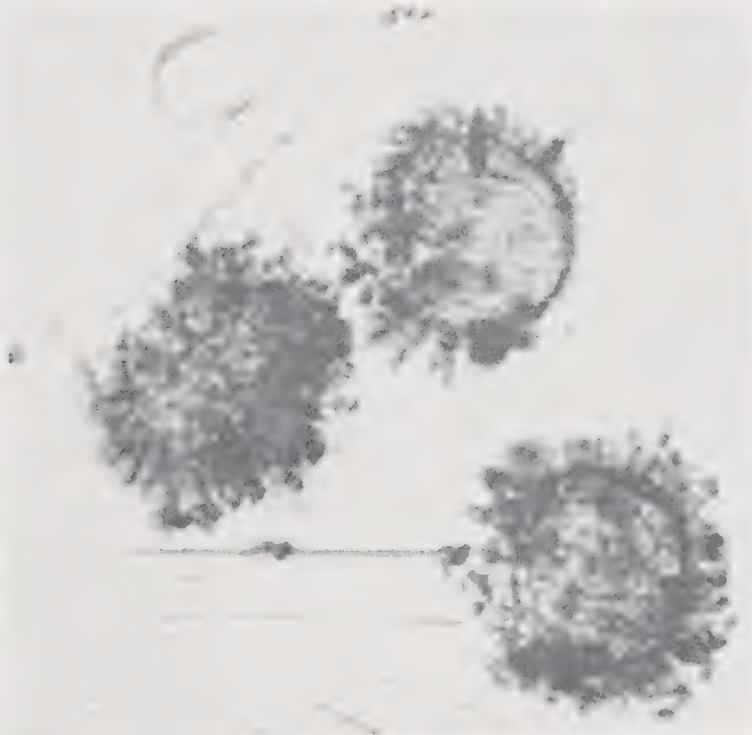


Fig. 5-4. Platinum micrograph of conidia and spore head of *Aspergillus niger*. (Courtesy of Eli Lilly and Co.)



around which the spores are located. When the sporangium is mature, the spores are liberated upon rupture of the sporangium wall, a portion of the latter and the columella generally remaining attached to the hypha. The spores of the terrestrial forms possess no means of locomotion and are commonly scattered by the wind or by insects. In the aquatic forms these asexual spores, known as zoospores, are generally flagellated. Endogenous spore formation is limited to the Phycomycetes, although a few members of this group produce exogenous spores rather than sporangiospores.

In other classes of Fungi, exogenous spores, *conidia* or *conidiospores*, are characteristically produced. The term conidia is generally employed although conidiospores would be more consistent with sporangiospores. The conidiospores are formed by constriction at the tip of fertile hyphae. The usual method is for a hypha to develop a constriction near the tip followed by a second constriction below the first, and the process is continued to form a chain of spores, the outermost conidium, which is the oldest, frequently also being the largest, at least until the spores are mature. The reverse type of spore formation is also observed, the first-formed conidium constricting to form another, etc., the outermost cell



Fig. 5-5. Photomicrograph of conidia of *Penicillium notatum*. (Courtesy of J. H. H. and C. J.)

in the chain being the youngest and frequently the smallest. In some species a conidiospore may form two buds, and the chain of immature spores in that manner becomes branched. In other species the conidiospores, or conidia, develop as buds from the mycelium or as a cluster rather than as a chain at the tip of the conidiophores. With maturity the conidia become separated and may be set free and scattered. The color exhibited by molds is for the most part due to the presence of pigments in the sporangiospores or conidiospores, the mycelium in the majority of species being colorless.

**Sexual Spore Formation.** With the exception of sporangiospore formation, the methods of asexual spore formation are not particularly characteristic of any class of Fungi, although types of spore-bearing structures or arrangements can be of help in finer classification. On the other hand sexual spore formation does vary characteristically with the different classes of Fungi. In the Phycomycetes, morphologically differentiated elements, a large female cell and a smaller male cell, fuse. The protoplasm of the female cell, an *oögonium*, contains a number of cells, each of which upon fertilization gives rise to an *oöspore*. This process of oöspore formation is *heterogamous*, morphologically distinct cells called *heterogametes* conjugating to form the fusion body. Since both the male and the female cells are produced on the same plant or thallus, the conjugation is said to be *homothallous*. In other species of Phycomycetes, morphologically similar cells may fuse to give rise to the production of *zygospores*. This process is therefore one of *isogamous* conjugation, but generally the cells which fuse arise from different plants (i.e., ones that have developed from different spores) of the same species, and conjugation is said to be *heterothallous*. While morphological differences may not be apparent, yet at times physiological differences between the conjugating strains can be noted and the strains are designated as *plus* or *minus* rather than as male or female.

In the Ascomycetes the sexual spores are formed within a cell or sac which when mature is called an *ascus*, and the spores are designated as *ascospores*. Ascospores are formed as a result of either heterothallous or homothallous conjugation, usually the latter. Likewise the process may be either isogamous or heterogamous, the latter type predominating. In most species two cells fuse, and the nuclei undergo three divisions with the ultimate production of eight ascospores within the ascus. This is in marked contrast to the formation of only one spore per fertilized cell as observed in the Phycomycetes. In some species, spore formation is not observed immediately after fusion, but instead a protective meshwork of new mycelium, a *perithecium*, is first formed. The ascospores develop in other hyphae originating from the original fertilized cell and contained in the perithecium.

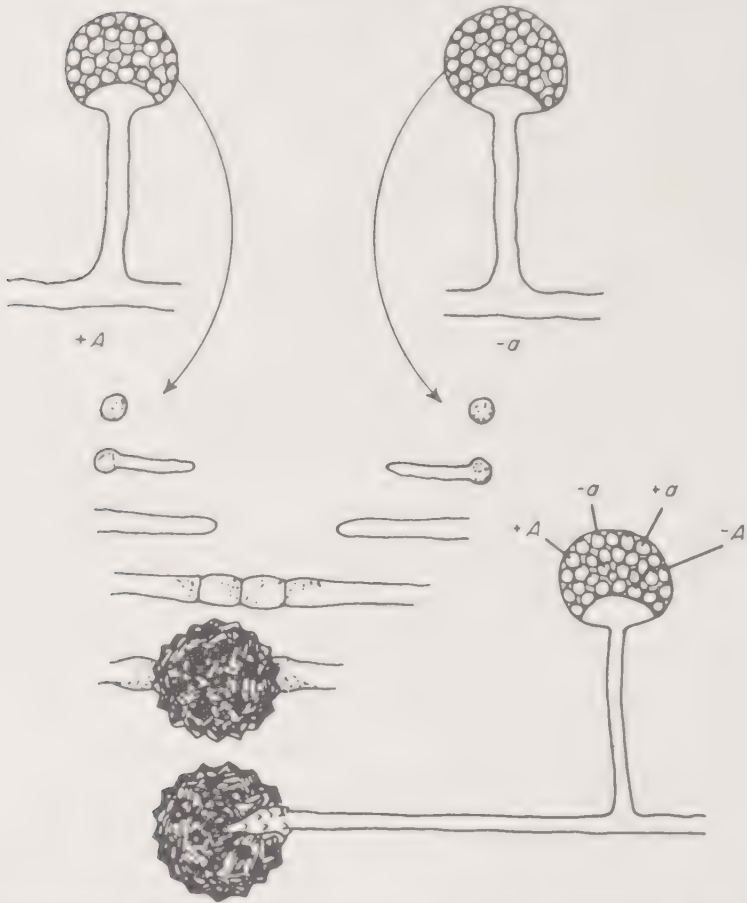


FIG. 5-6. Schematic illustration of conjugation of fungi. [*Genetics of the Fungi*, by C. Lindegren, *Annual Review of Microbiology*, **2**, 48 (1948).]

In the Basidiomycetes sexual spore formation is heterothallic and isogamous. The two nuclei in the fusion cell do not fuse, and instead an extensive binucleate mycelium develops from the fusion cell. The tips of the hyphae so produced become expanded to form characteristic club- or pear-shaped bodies known as *basidia*. The nuclei fuse within the basidium and undergo two divisions to yield four nuclei. Four little stems, or sterigmata, arise from the end of a basidium; one nucleus enters each stem and is finally present in the basidiospore, which develops at the end of a sterigma. When mature, the basidiospores may be forcibly discharged. Basidiospores are the only reproductive bodies found in the mushrooms, puffballs, and bracket fungi, while in parasitic basidiomycetes such as the rusts and smuts several other types of spore formation can also be observed. The Basidiomycetes are relatively large in comparison with the other fungi and will not be considered further.

The majority of the Fungi Imperfecti multiply by means of conidio-

spores although oidia or chlamydospore formation is also observed. A few species produce sporangiospores and are probably Phycomycetes, or derived therefrom, but their method of sexual reproduction is unknown. The majority may have been Ascomycetes which lost their power of sexual reproduction; at least ascospore formation has not been observed. Many of the fungi employed industrially and others which are pathogenic to man and animals are grouped in this class. Actually it is a very heterogeneous group of fungi, a catchall for those which do not fit into the first three classes, and in many instances, as Henrici has stressed, it is our knowledge of these fungi, rather than the organisms themselves which is imperfect.

**The Common Molds.** We have seen that the Phycomycetes are characterized by a nonseptate mycelium, asexual sporangiospores borne in a sporangium, and sexual spore formation—in the aquatic forms by heterogamous, homothallic conjugation; in the terrestrial forms by isogamous, heterothallic fusion; in either case the fusion body giving rise to only one spore, an oöspore or zygosporangium, respectively. Familiar examples are the water mold, *Saprolegnia*, which is frequently encountered as a whitish scum upon fish which have been injured or carelessly handled by the fisherman; the common black bread mold, *Rhizopus nigricans*; the coarse, woolly, white "manure mold," *Mucor mucedo*, and similar species commonly found on decaying organic matter; and *Phytophthora infestans*, the causative agent of a highly destructive disease of potatoes known as late blight. Schematic illustrations of *Rhizopus* and of *Mucor* species are presented in Figs. 5-7 and 5-8, respectively. The main distinction between these genera is that in the genus *Rhizopus*, the spore-bearing hyphae arise in clusters from the nodes of runners or stolons, while the sporangiophores of *Mucor* arise singly at any point in the mycelium.

The Ascomycetes are characterized by a septate mycelium, asexual conidia (conidiospores) attached to the hyphae rather than enclosed in a sporangium, and a number of sexual spores, usually eight, produced within an ascus as the result of the fusion of a pair of either iso- or heterogametes in homo- or heterothallic conjugation, depending upon the species. The two most common genera are *Aspergillus* and *Penicillium*, species of which are frequently observed on decaying fruits and vegetables. The aspergilli are greenish, yellow-brown, or black fungi which bear exogenous asexual conidiospores in chains extending from the ends of a large number of little stalks, sterigmata, arising from a club-like swelling, the *vesicle*, at the end of fertile hyphae. These conidiophores are nonseptate and arise from specialized, swollen foot cells in the septate mycelium. Penicillia are similar in appearance, but the conidiophores do not arise from specialized cells, are septate and end in a whorl



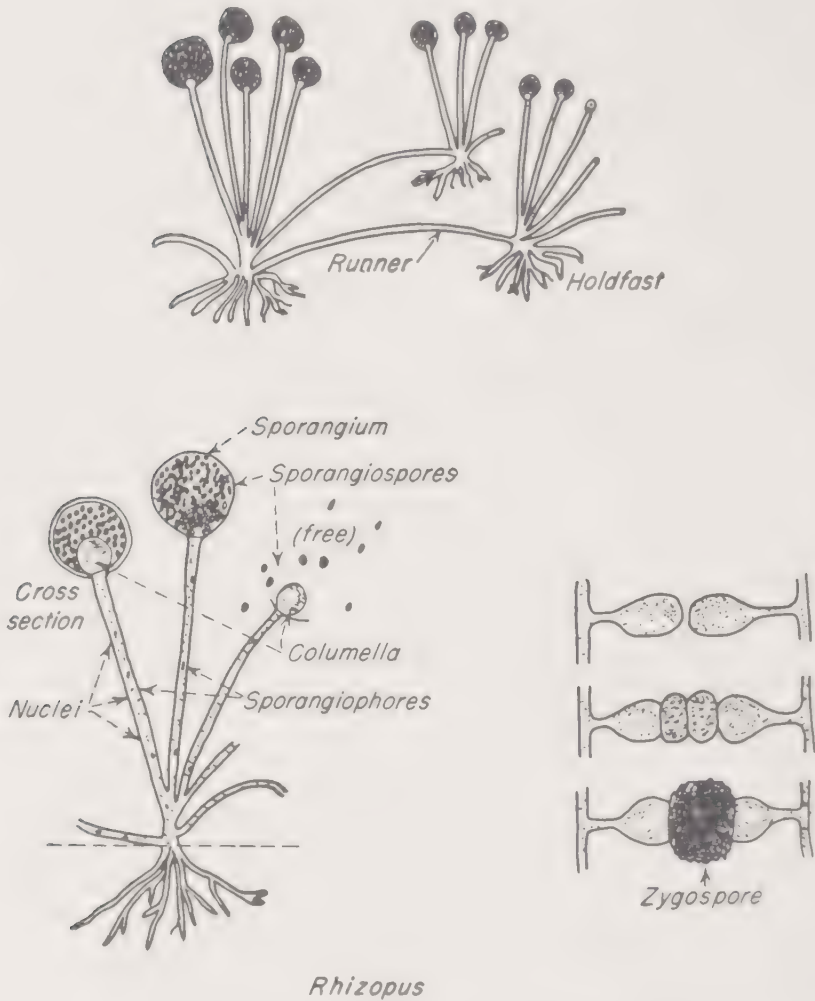


FIG. 5-7. Schematic illustration of the genus *Rhizopus*.

of short branches, *metulae*, each of which bears another whorl of smaller branches, *sterigmata*, from which the chains of conidia extend. The spore heads of *Penicillium* species appear much looser than those of *Aspergillus* and are brush-like in appearance, the name *Penicillium* indicating a brush-like appearance. *Aspergillus niger* is the most common species and is frequently encountered as a contaminant in the laboratory, its large, very black, globular spore heads being quite characteristic. *Penicillium expansum*, a blue-green mold frequently observed on the skin of apples and citrus fruits, is a common species. Other species of general interest are *P. roqueforti* and *P. camemberti*, important in the ripening of the cheeses suggested by their names, and *P. notatum* and related species employed in the production of the important antibiotic and chemothera-

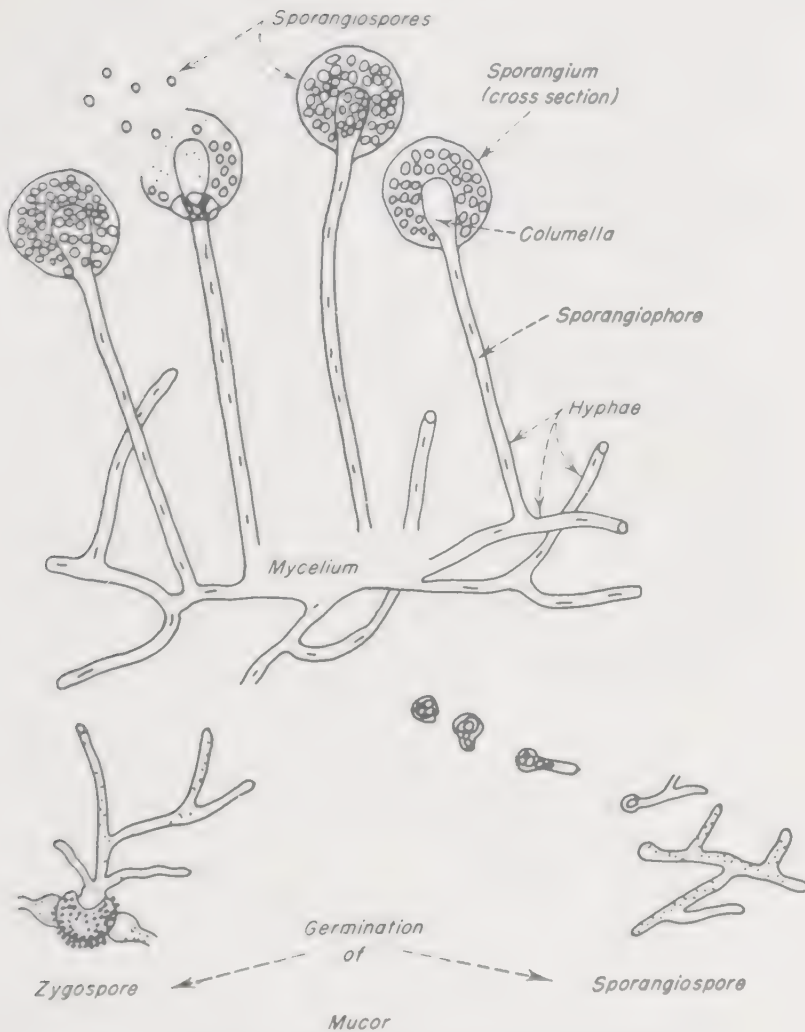


FIG. 5-8. Schematic illustration of the genus *Mucor*.

peptide agent penicillin. Schematic representations of these two genera are presented in Fig. 5-9.

The class Fungi Imperfecti is a heterogeneous collection of fungi which did not fit into the other classes. This makes the classification of fungi in this group very difficult, but confusion also exists in the classification of the more "perfect" fungi. Only the more apparent structures of the fungi have been discussed, and classification into the finer groups frequently rests on relatively minute differences between these structures in different species.

The mold can be observed more readily than the bacteria under the microscope since they are larger and since staining is not essential for observation of most morphological details involved in general classifica-

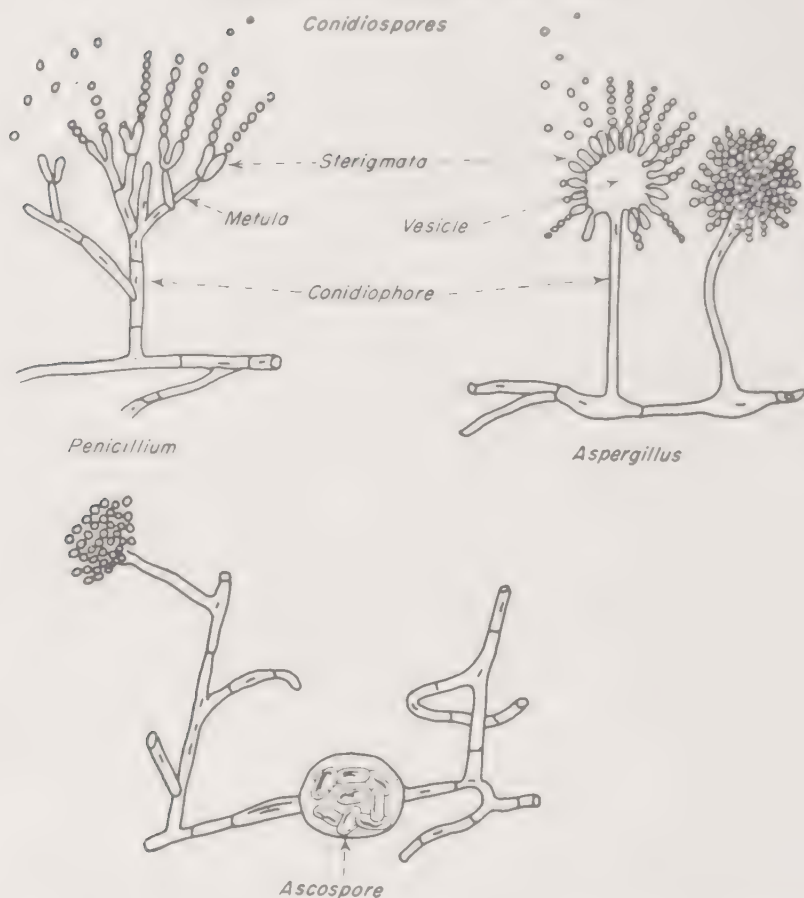


FIG. 5-9. Schematic illustration of the genera *Penicillium* and *Aspergillus*.

tion. Stained smears or unstained preparations are of little value for microscopic examination as they generally consist only of aerial hyphae and spores. It is much better to grow the molds in a hanging drop or on agar on a microscope slide prepared in the manner described by Henrici or in the more complex culture chamber for microscopic examination devised by Brown. Observations on the growing plants can be made at frequent intervals, and the entire growth is available for examination without injury to the fungus.

Molds differ from the bacteria not only in morphology but also in many of their physiological properties. They tend to grow more slowly than the bacteria. In general their ability to utilize complex organic matter such as cellulose for food greatly exceeds that of the bacteria, and they frequently thrive under conditions of acidity or of osmotic pressure that are inhibitory to most bacteria. The majority are saprophytic in nutrition and are important scavengers in the disposal of waste products, in particular of the complex matter in the dead bodies of plants and to a



FIG. 5-10. Henrici slide culture of *Macor macedo* under high power.

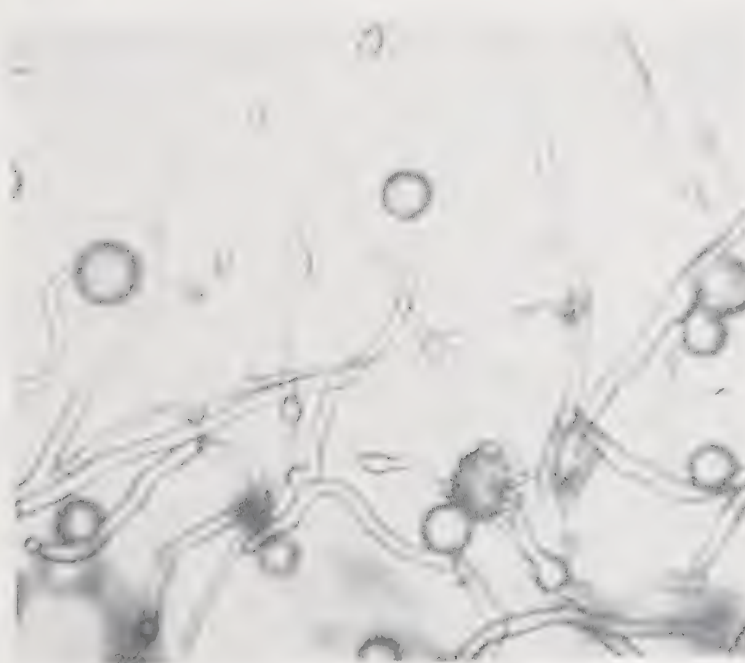


FIG. 5-11. Henrici slide culture of *Aspergillus niger* under high power.





FIG. 5-12. Hemmer slide culture of *Penicillium notatum* under high power.



FIG. 5-13. Photomicrograph of fungi in a page of an old book. (Courtesy of W. H. Sumner.)

lesser extent of animals. While they are frequently found growing in damp, dark places, they require less free moisture for growth than the yeasts and bacteria. Hence they are able to grow on and bring about the decomposition of leather, paper, or cloth in a fairly humid environment. Also, since they tolerate high osmotic pressures, they are frequently found growing on the surface of jams, jellies, the brines employed in pickle production, or on salted meats. Such growth so alters the material upon which it occurs that bacteria may multiply as secondary invaders, frequently with more marked damage to the material. The problem of spoilage of dried fruits by molds is of considerable importance, a more tender product being obtained if the moisture content is not too low. However, if the moisture content is much above 15 per cent, mold growth soon becomes apparent. Therefore a balance must be struck between the two factors, possible growth of molds and the texture of the fruit, and incidentally, selling price, since the higher the moisture content, the greater the financial return. Contamination of dried fruit by mold spores would be impossible to prevent since they are so abundant in the air, a fact readily demonstrable on incubation of nutrient media exposed for a few minutes to the air.

**Keys for the Identification of Common Molds.** A detailed classification of the molds would be too difficult to consider in a text on general bacteriology. Bacteriologists have found it convenient to construct keys, based on general morphological considerations, for the rapid identification of the more common molds. The following key for the differentiation of the more common genera of molds is based on one devised by Buchanan in which the molds are all grouped as Hyphomycetes and divided into families and genera on the basis of asexual spore formation.

**KEY FOR IDENTIFICATION OF THE COMMON MOLDS<sup>1</sup>**  
(AFTER BUCHANAN)

- I. Sporangiospores borne in sporangia. Nonseptate mycelium
  - A. Sporangiohores arising in clusters from nodes of runners or stolons..... *Rhizopus*
  - B. Sporangiohores arising singly, runners or stolons not formed..... *Mucor*
- II. Conidiospores (conidia) borne exogenously
  - A. Conidiophores usually separate, not united into definite masses
    - 1. Neither conidia nor hyphae smoky or dark in color
      - a. Conidia one celled
        - aa. Formed as oïdia..... *Oöspora*
        - bb. Formed in chains radiating from the swollen tip of a conidiophore..... *Aspergillus*

<sup>1</sup> E. D. Husted and R. E. Buchanan "Bacteriology," The Macmillan Company, New York, 1938.

## KEY FOR IDENTIFICATION OF THE COMMON MOLDS (Cont.)

- cc. Formed in chains radiating from branching, brush-like conidiophores ..... *Penicillium*
- b. Conidia two-celled, pear-shaped ..... *Trichothecium*
2. Conidia and/or hyphae smoky or dark in color
- a. Conidia one-celled, in branched chains ..... *Hormodendrum*
- b. Similar to *Hormodendrum* but conidia become two-celled in old cultures ..... *Cladosporium*
- c. Conidia many-celled, in chains ..... *Alternaria*
- B. Conidiophores united into definite masses
1. Neither conidia nor hyphae smoky or dark ..... *Isaria*
2. Conidia and hyphae smoky or dark ..... *Stysanus*

Other keys have been prepared with particular reference to the needs and interests of an individual or group. One key, listing the more common species of yeasts and molds pathogenic for man together with the main characteristics of the infection produced, may be represented as follows:

## KEY TO THE PRINCIPAL GENERA AND SPECIES OF FUNGI PATHOGENIC FOR MAN

## Infection

- I. Multicellular, filamentous forms
- A. Ringworm fungi, dermatophytes
1. *Microsporum*, unbranched septate mycelium containing small rounded spores
- canis, audonini, gypseum* ..... Scalp ringworm (tinea capitis)
2. *Trichophyton*, branching mycelium with chains of spore-like elements
- schoenleini, mentagrophytes, tonsurans* ..... Ringworm of scalp and skin
3. *Epidermophyton*, septate mycelium, terminal clavate *fuseaux*
- floccosum* ..... Mycosis of hands, feet, and groins
- B. Aspergillosis
1. *Aspergillus*, septate mycelium, conidiospores form around enlarged end of conidiophore
- fumigatus* ..... Lung infection of birds, rare in man
- II. Filamentous fungi, at times unicellular, yeast-like forms
1. *Coccidioides*, spherical forms in tissue and pus, no budding occurs, mycelial filaments during aerobic growth
- immitis* ..... Coccidiomycosis (Valley fever), infection of lungs. Coccidioidal granuloma, ulcerative

## KEY TO THE PRINCIPAL GENERA AND SPECIES OF FUNGI PATHOGENIC FOR MAN (Cont.)

## Infection

2. *Sporotrichum*, oval or cigar-shaped bodies in pus, septate mycelium in laboratory<sup>1</sup> media  
*schenckii* ..... Sporotrichosis [infection of skin, subcutaneous tissue, and lymphatics (red nodules)]
  3. *Candida* (*Monilia*), small, oval, thin-walled, budding cells associated with mycelial elements  
*albicans* ..... Thrush (a mycosis of mucous membranes). May also cause an infection of the skin or lungs
  4. *Blastomycetes* (*Oidium*), in tissues, yeast-like cells; in cultures, mycelial growth and ascospore formation  
*dermatitidis* ..... Blastomycosis (suppurative and granulomatous lesions of skin and lungs)
- III. No filaments or mycelia
1. *Cryptococcus* (*Torula*), spherical to ovoid cells. No ascospores  
*neoformans* ..... Cryptococcosis [mycosis of skin, lungs, brain, or meninges (meningitis)]

## THE YEASTS

The term yeast is used with different meanings, the most common one referring to unicellular forms somewhat larger than the bacteria which do possess a readily demonstrable nucleus, which multiply by budding, and which characteristically produce considerable amounts of ethyl alcohol and carbon dioxide from fermentable sugars. Such a concept holds true for only a limited number of forms regarded as yeasts by the biologist. We have mentioned that the terms molds and yeasts have no taxonomic significance, and organisms considered to be yeasts are classified in the Ascomycetes, the Basidiomycetes, and the Fungi Imperfecti. Not all these forms are unicellular; some multiply by fission rather than by budding, and some produce little or no alcohol and gas. The most satisfactory definition of yeasts as a group is a modification of an earlier definition by Henrieci which may be restated as *yeasts are fungi with readily demonstrable nuclei with which the usual and dominant or most characteristic growth is unicellular*. This definition is not without its faults but will serve in a general way.



The majority of the yeasts appear to belong to the Ascomycetes or to be derived from the Ascomycetes with loss of ability to form ascospores; at least in many instances ascospore formation has not been observed. Classification of the yeasts depends to a considerable extent upon cytology and methods of reproduction and to a lesser extent than with the bacteria upon biochemical characteristics.

Yeasts were described rather completely in 1680 by Leeuwenhoek, who recognized them as oval or spherical, globular bodies commonly present in fermenting liquids. By 1839 various workers had reported that beer and wine yeasts multiply by budding, and in that year Schwann observed spores within yeast cells. The studies of Pasteur gave final proof that alcoholic fermentation is the result of the metabolic activities of yeasts.

The yeast cell averages 4 to 5  $\mu$  in diameter and possesses the usual cellular structures, a rather rigid cell wall composed of cellulose or cellulose-like material surrounding the cytoplasm, which is enclosed by a cell membrane. Various granules and vacuoles can be observed in the cytoplasm, the granules generally being glycogen or iogen, volutin, and droplets of fat. The vacuoles are rather refractile bodies apparently filled with liquid material and are most evident in older cultures of yeast approaching starvation conditions. The nucleus is relatively large in comparison with the size of the cell and undergoes division with cell division, one-half remaining in the mother cell and one-half passing into the daughter cell. A daughter cell can form a bud before it becomes detached from the original cell, and in this manner chains of cells or a branching, filamentous-like structure may be produced.

**Sexual Reproduction.** The fact that yeast can multiply by sexual means was definitely established by 1902 as a result of the studies of Guilliermond and Barker. Guilliermond showed that the formation of ascospores by *Schizosaccharomyces octosporus* was preceded by the conjugation of two like cells, little protuberances from each uniting to form a copulation canal. The contents of the two cells mixed to form a zygospore, the nucleus of one cell fusing with that of the second. This is an example of isogamic conjugation, since the two gametes are nearly equal in size. In other species or genera, heterogamic conjugation has been observed. The zygospore develops, and its fusion nucleus divides, giving rise to four, and in a few species to eight, daughter nuclei contained in the ascus. Spore formation in yeasts is a method of reproduction, since the spores developing in one zygospore can give rise to four or eight vegetative cells. This is a differential characteristic between the yeasts and the bacteria, in the latter only one spore being formed per cell.

In a large number of yeasts, particularly in those of industrial importance, the *Saccharomyces*, ascospores are formed without any evidence of conjugation preceding spore formation. These yeasts were considered



FIG. 5-14. Photomicrograph of *Saccharomyces cerevisiae* (bread yeast) suspended in 1:20,000 crystal violet.

to be parthenogenic, or devoid of sexuality. In some species copulation tubes do form, but they fail to fuse. In other species four ascospores are formed within a cell without evidence of previous conjugation, but on germination the spores conjugate in pairs within the ascus, and only two vegetative cells are produced from them instead of four. Guilliermond designated this process as parthenogamy. He considered these various aspects of ascospore formation or fusion to be an indication of retrograde evolution in yeasts, the various stages of decrease in sexuality being from true conjugation through parthenogamy and parthenogenesis to the complete loss of the ability to form ascospores.

Winge and Lausten in 1935 and subsequent years demonstrated that ascospore formation and germination in the genera *Saccharomyces* and *Saccharomyces* are associated with a separation of genes that makes the spores in the ascus differ genetically. Since ascospores conjugate, as mentioned above, this process results in fertilized cells of genetic composition different from the parent cell, even though the parent cell was from a culture originally derived from a single cell. Or, starting with spores from different species, crosses were produced when these spores fused, and the hybrids which resulted had characters different from those of the two parent strains by themselves. Winge and Lausten developed microma-

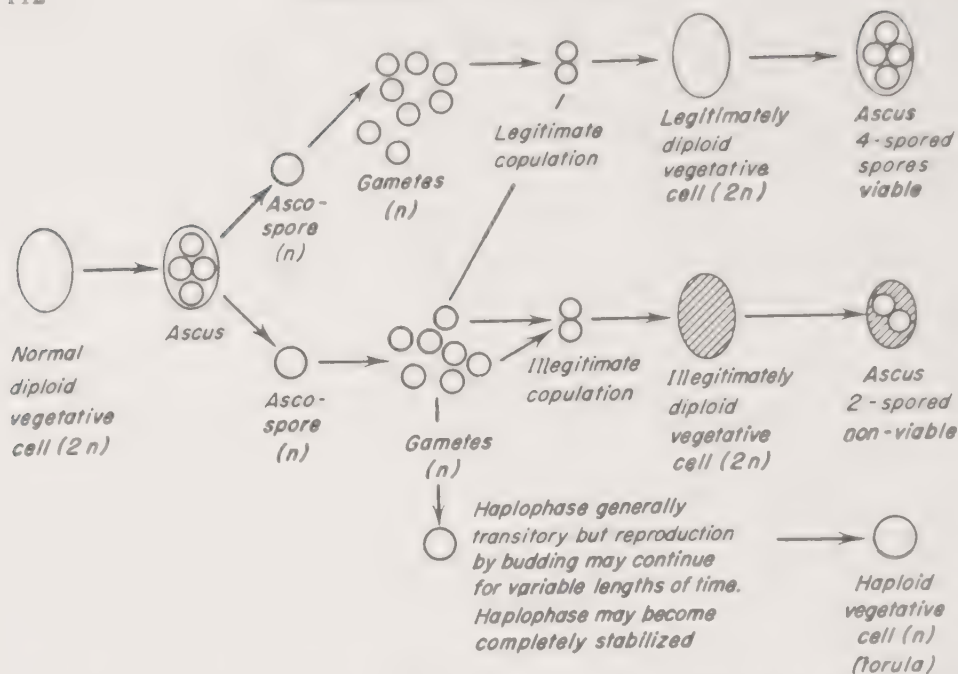


FIG. 5-15. Life cycle of *Saccharomyces cerevisiae*. [Genetics of the fungi, by C. Lindgren, *Annual Review of Microbiology*, 2, 58 (1948).]

nipulation techniques for the isolation of the individual spores from the ascus and for the cultivation of strains from each spore. This provided an excellent method for the study of hybrid formation. Hybridization of yeast in this manner suggests that hybrids can be produced in which the desirable characters of several species may ultimately be combined into a new strain, as in the breeding of new plant and animal strains.

Lindgren extended the studies of Winge and Lausten and concluded that the large, vegetative, ellipsoidal cells of yeasts are diploids, i.e., contain the full complement of chromosomes. These "normal" cells produce viable four-spored asci. These ascospores on germination produce smaller, rounder, and generally less active cells which are haploids, the nuclei containing only one-half the normal number of chromosomes. Two small, haploid cells fuse to form a characteristic large diploid vegetative cell, the descendants of which tend to maintain constant genetic characteristics when cultivated under conditions inimical to ascospore formation. The life cycle just described starts anew when sporulation occurs.

Various observations with regard to sexual behavior indicate that the vegetative cells of one group of yeasts, the genera *Schizosaccharomyces*, *Zygosaccharomyces*, *Zygopichia*, *Debaryomyces*, *Nadsonia*, and *Nematospira*, are haploid, while the vegetative cells of *Saccharomyces*, *Saccharomyces*, and *Hansenula* are ordinarily diploid, being haploid only



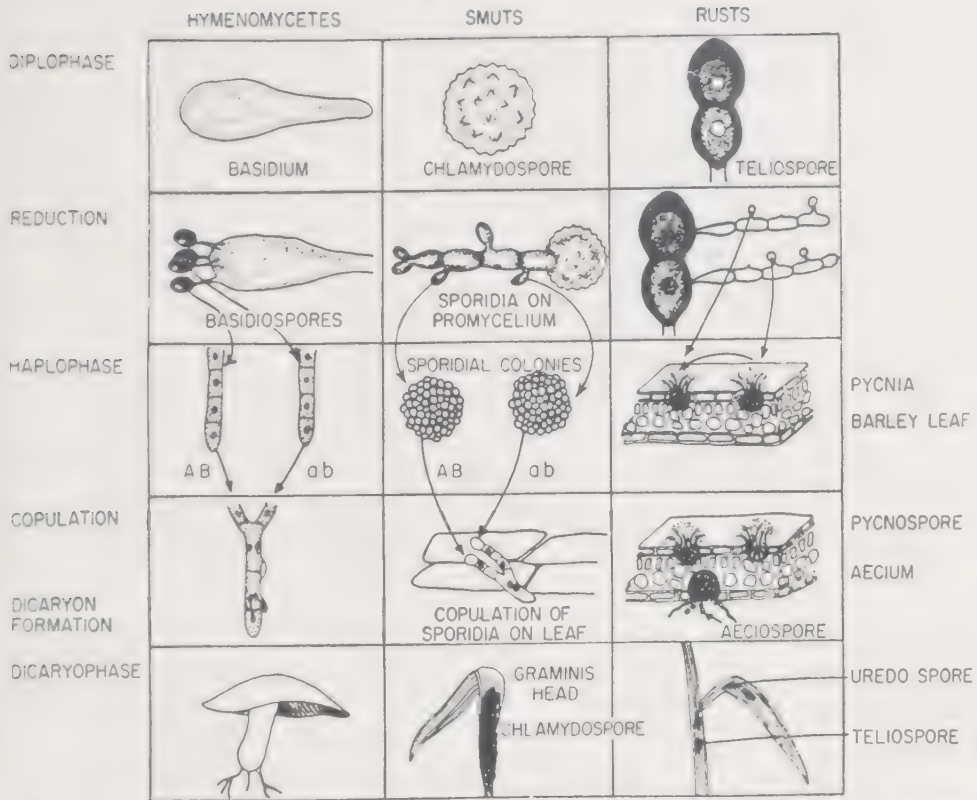


FIG. 5-16. Life cycles of higher fungi. [*Genetics of the fungi*, by C. Lindgren, *Annual Review of Microbiology*, 2, 51 (1948).]

in the spore stage and in the small cells which arise from the spores. A third and very miscellaneous group would include all those forms not known to produce ascospores.

**Classification of Yeasts.** An entirely satisfactory classification of the yeasts has not been developed. Some forms generally considered to be yeasts do produce mycelia and are intermediate between the yeasts and the molds. Others do not produce ascospores and therefore do not belong in the true yeasts. Most of these various forms, however, have many characteristics in common and are therefore frequently classified together. Keys have been developed for the classification of yeasts of industrial or pathogenic importance, such a key for the more common pathogenic yeasts (and molds) was presented on pages 108-109. A common classification of industrial yeasts is as top or bottom fermenters and as alcohol, wine, or bread yeasts, depending upon their tendency to remain near the top of, or to settle out from, a fermenting liquid and the purpose for which the yeast is best suited. A classification of the ascosporeogenous yeasts according to Stelling-Decker and of the false yeasts as devised by Lodder is presented below in forms developed by Henrici.



CLASSIFICATION OF YEASTS<sup>1</sup>

(AFTER HENRICI)

*Family Endomycetaceae*

Growth forms: mycelium, pseudomycelium, oidia, or yeast cells ("conidia"), together or singly. Vegetative multiplication by transverse fission or by budding. Naked asci result from isogamous or heterogamous conjugation or parthenogenetic. Ascospores spherical, hemispherical, angular, sickle- or spindle-shaped, smooth, warty, or with an encircling rim. Both oxidative and fermentative species.

Subfamily A. *Eremascoideae*. Growth form only mycelium. Vegetative multiplication by transverse fission. Spores hat-shaped, result from isogamous conjugation. Dissimilation exclusively oxidative. But one genus—*Eremascus*.

Subfamily B. *Endomycoideae*. Growth form either mycelium with oidia or only oidia. Vegetative multiplication by transverse fission. Spores round, oval, or hat-shaped, result from isogamous or heterogamous conjugation. Dissimilation oxidative or fermentative. There are two genera—*Endomyces* with both mycelium and oidia, and both oxidative and fermentative respiration; and *Schizosaccharomyces* with no mycelium, only oidia multiplying by transverse fission and with dominantly fermentative respiration.

Subfamily C. *Saccharomycoideae*. Growth form either mycelium with yeast cells ("conidia"), occasionally also oidia, or only budding yeast cells and then often pseudomycelium. Vegetative multiplication by transverse fission, by multipolar budding or by bipolar budding, the latter upon a broad base. Spores spherical, hemispherical, angular or sickle-shaped, or with an encircling ridge, formed by isogamous or heterogamous copulation or parthenogenetic. All transitions between oxidative and fermentative dissimilation. There are three tribes in this subfamily:

Tribe A. *Endomycopseae*. Growth form mycelium with buds ("conidia"), at times oidia. Vegetative multiplication by transverse fission and by multipolar budding. Spores parthenogenetic or following isogamous copulation. But one genus—*Endomycopsis*. Dissimilation is dominantly oxidative, at times also fermentative.

Tribe B. *Saccharomycetaceae*. No mycelium, only budding yeast cells or pseudomycelium. Vegetative multiplication by multipolar budding. Spores produced by isogamous or heterogamous conjugation, or parthenogenetic. This tribe includes the largest number of species of spore-forming yeasts, contained in the genera *Saccharomyces*, *Torulaspora*, *Pichia*, *Hansenula*, *Debaryomyces*, and *Schwanniomyces*.

Tribe C. *Nadsoniae*. No mycelium, only budding yeast cells, at times pseudomycelium. Vegetative multiplication by bipolar budding, more or less upon a broad base. Spores parthenogenetic or following heterogamous conjugation. There are three genera: *Saccharomycodes* with round spores that conjugate during germination, *Hanseniaspora* with parthenogenetic spores, and *Nadsonia* in which following heterogamous conjugation between a bud and the mother cell, a second bud develops into an ascus.

Subfamily D. *Nematosporaceae*. Growth form mycelium and budding yeast cells. Vegetative multiplication by multipolar budding. Spores needle- or spindle-shaped, with or without flagella, parthenogenetic or formed after isogamous conjugation. Both oxidative and fermentative. There are three genera: *Mono-*

<sup>1</sup> Henrici, *Bacteriological Reviews*, 5, 97 (1941).

*sporalla* with one needle-shaped spore; *Nematospora* with two to eight flagellated spindle-shaped spores produced parthenogenetically; and *Coccidiascus* with eight spindle-shaped nonflagellate spores produced by isogamous conjugation.

The Torulopsidoideae comprise the majority of the "wild yeasts," common as contaminants in all sorts of bacteriological work.

## KEY TO THE GENERA OF TORULOPSIDOIDEAE

1. *a.* Cells mostly lemon-shaped, bipolar budding..... *Kloeckera*  
*b.* Cells mostly triangular, budding at the three angles..... *Trigonopsis*  
*c.* Cells mostly flask-shaped, budding often upon a broad base *Pityrosporum*  
*d.* Cells otherwise, usually round, oval or cylindrical
2. *a.* No pellicle in wort cultures, or only a soft slimy pellicle after some time  
*b.* A matte, dry pellicle in wort cultures from the beginning
3. *a.* Formation of long slender tubular processes resembling the copulation tubes of *Zygosaccharomyces* on Gorodkova agar..... *Asporomyces*  
*b.* No tubular processes formed..... *Torulopsis*
4. *a.* Cells often cylindrical, multiplication by budding, the buds not separated from the mother cell by fission..... *Mycoderma*  
*b.* Cells polymorphous, multiplication by budding, the buds often separating from the mother cell by fission..... *Schizoblastosporium*

## CHAPTER 6

### THE BACTERIA

In the previous chapter the true fungi, the Eumycetes, were considered in a general manner. The slime molds, or Myxomycetes, and the fission fungi, or Schizomycetes, the bacteria, are related to the true fungi, and they are frequently considered as pseudo fungi, or *Pseudomycetes*. The Myxomycetes will not be discussed further, and at this time a general consideration of the various orders and families of bacteria, together with the genera of certain of the less common families, will be developed.

Some of the organisms commonly included under the term bacteria show marked resemblances to certain of the true fungi, others to the blue-green algae, others to various protozoa, and still others appear to be more or less independent forms of life. The bacteria are considered by some to be a fairly homogeneous group of primitive forms of life from which higher forms have developed; by others to be the result of retrograde evolution from higher microorganisms. Arguments and evidence in support of both views can be advanced, but for our purpose it is sufficient to consider the bacteria as a rather heterogeneous collection of somewhat related forms primarily characterized by their small size and relatively undifferentiated structure.

The bacteria are commonly classified in the plant kingdom. The fact that bacteria are assigned to families, orders, and classes does not mean that these groups have the significance they have in the classification of plants and animals, where phylogenetic considerations can be employed to a greater extent.

In the higher plants differentiation and classification are based almost entirely upon morphology—size, shape, arrangement, structure, and color of the constituent parts—while in the true fungi and to a greater extent in the bacteria such considerations do not lead very far into an adequate classification. At an early date bacteriologists realized that a classification based on morphology alone was inadequate, a fact recognized in the eighteenth century by the botanist Linnaeus who assigned the bacteria to the order *chaos*. Staining properties and biochemical reactions were introduced as supplementary aids in classification, and at the present time

bacteria are classified to a great extent on the basis of *what they do* rather than *what they look like*. We will consider primarily the orders and families of bacteria at this time. An example of the details employed in the classification of bacteria into species and genera in one family, the Enterobacteriaceae, will be presented in Chap. 22. Both physiological and serological characteristics, to be considered later, are employed in this finer classification.

A system of classification of plants based upon the developmental history, or phylogeny, of the individual species was developed by the 1870's. Nothing was known of the developmental history of bacteria in 1872 when Ferdinand Cohn recognized that an attempt must be made to ensure a stable nomenclature for the various bacteria then known or being discovered with increasing rapidity. He adequately described a number of species and allocated them to six genera on the basis of morphology. He recognized that the genera as employed in his classification were not equivalent in significance to the concept of a genus in plant and animal biology. Nothing better could be accomplished at a time of relative ignorance concerning the nature and properties of the bacteria.

With increasing knowledge of the structures and activities of bacteria and with descriptions of many new species appearing, it became apparent that Cohn's system of classification was far from adequate. Migula in 1895 to 1900 attempted to integrate the newer knowledge into a more complete system of classification than the one proposed by Cohn. Migula divided the bacteria into two orders, one of which, the Thiobacteria, or sulfur bacteria, was created primarily on the basis of the unique physiology of these organisms. Little attention, however, was paid to physiological differences between the bacteria as an aid in differentiation.

In contrast to the earlier attempts at classification, Orla-Jensen in 1909 proposed a system based primarily upon physiological considerations of the bacteria, although morphological considerations were also employed. An attempt was made to trace the phylogenetic development of bacteria, based on the premise that the first bacterium was an autotrophic organism, it being assumed that organic matter was lacking at an early date in the history of the earth. Such an organism would have to have enormous synthetic powers! Modern concepts suggest that organic matter in certain forms was present early in the history of the world and that primitive life could have developed more readily from it than from inorganic matter. This hypothesis raises serious questions concerning Orla-Jensen's basic premise and leads to the suggestion that autotrophic species may have developed from heterotrophic ones by retrograde evolution, rather than having been the original type of bacteria.

At the present time there is no good evidence as to which bacterium,



or ancestor of the bacteria known today, could have been the original form of bacterial life. Interrelationships can be traced between many bacteria, but as yet phylogenetic considerations do not lead very far in tracing the evolution of bacteria and in the development of an entirely adequate system of classification. Possibly the most unsatisfactory development is that the "form genera" of Cohn and the "physiological genera" of Orla-Jensen are more or less merged with each other, and the product is accepted by many as equivalent to the concept of genera employed in botany and zoology. Considerations of this nature may appear out of place in an elementary text, but the student should realize from the start that the systems of classification commonly employed in bacteriology are based to a great extent on expediency rather than on actual relationships. This understanding can save future trouble for those who continue in the study of the bacteria. Too little is known about bacteria to permit the development of an entirely satisfactory classification.

Any system of classification is only a tool to be used in understanding the relationships of different organisms and to provide keys for the identification of the different forms of life. A knowledge of life cycles, distribution, development, and structure of the organism to be classified or identified is essential, and these various factors considered together indicate where the organism fits into the general scheme. Such considerations do not lead very far as yet in the classification of bacteria, and any classification proposed for these organisms must be tentative and subject to change with increase in knowledge concerning the constituents of the proposed system.

The student in bacteriology will frequently find that different names are applied to the same organism by different workers and that different classifications are employed. There is no universally accepted classification and nomenclature, but the one most commonly employed in the United States is a development from a system proposed by a committee of the Society of American Bacteriologists under the chairmanship of D. H. Bergey. In its original form the classification was based to a considerable extent upon suggestions made by R. E. Buchanan and also upon the work of the earlier systematists. This system has been revised a number of times, and the nomenclature for genera and species employed in this text is from the sixth edition of "Bergey's Manual of Determinative Bacteriology" (edited by R. S. Breed, A. P. Hitchens, and E. G. D. Murray, published by The Williams & Wilkins Company, Baltimore). The seventh edition is now (1956) in preparation, and an outline of the major taxa will be presented for comparison and reference.

In the sixth edition of Bergey's Manual the class *Schizomycetes* is defined and divided into five orders as follows:

CLASS SCHIZOMYCETES<sup>1</sup>

Typically unicellular plants. Cells usually small, sometimes ultramicroscopic. Frequently motile. As in the closely related blue-green algae (class *Schizophyceae*), the cells lack the definitely organized nucleus found in the cells of higher plants and animals. However, bodies containing chromatin which may represent simple nuclei are demonstrable in some cases. Individual cells may be spherical; or straight, curved, or spiral rods. These cells may occur in regular or irregular masses or even in cysts. Where they remain attached to each other after cell division, they may form chains or even definite filaments. The latter may show some differentiation into holdfast cells, and into motile or nonmotile reproductive cells (conidia). Some grow as branching mycelial threads whose diameter is not greater than that of ordinary bacterial cells, i.e., about one micron. Some species produce pigments. The true purple and green bacteria possess pigments much like or related to the true chlorophylls of higher plants. These pigments have photosynthetic properties. The phycocyanin found in the blue-green algae does not occur in the Schizomycetes. Multiplication is typically by cell division. Endospores are formed by some species included in Eubacteriales. Sporocysts are found in Myxobacteriales. Ultramicroscopic reproductive bodies are found in Borrelomycetaceae. The bacteria are free-living, saprophytic, parasitic, or even pathogenic. The latter types cause diseases of either plants or animals. Five orders are recognized.

## KEY TO THE ORDERS AND SUBORDERS OF THE CLASS SCHIZOMYCETES

- A. Cells rigid, not flexuous. Motility by means of flagella or by a gliding movement.  
 1. Cells single, in chains or masses. Not branching and mycelial in character. Not arranged in filaments. Not acid fast. Motility when present by means of flagella.

*Order I. Eubacteriales*

- a.* Do not possess photosynthetic pigments. Cells do not contain free sulfur.  
*b.* Not attached by a stalk. Do not deposit ferric hydroxide.

*Suborder I. Eubacteriineae (True Bacteria)*

- bb.* Attached to substrate, usually by a stalk. Some deposit ferric hydroxide.

*Suborder II. Caulobacteriineae (Stalked Bacteria)*

- aa.* Possesses photosynthetic chlorophyll-like pigments. Some cells contain free sulfur.

<sup>1</sup> Description of the Schizomycetes and the key to orders and suborders reproduced by permission of The Williams & Wilkins Company and the editors.

KEY TO THE ORDERS AND SUBORDERS OF THE CLASS SCHIZOMYCETES (*Continued*)*Suborder III. Rhodobacteriineae (Photosynthetic Bacteria)*

2. Organisms forming elongated usually branching and mycelial cells. Multiply by cell division, special spores, endospores, and conidia. Sometimes acid fast. Nonmotile.

*Order II. Actinomycetales (Thread-like or Filamentous Bacteria)*

3. Cells in filaments frequently enclosed in a tubular sheath with or without a deposit of ferric hydroxide. Sometimes attached. Motile flagellate and non-motile conidia. Filaments sometimes motile with a gliding movement. Cells sometimes contain free sulfur.

*Order III. Chlamydobacteriales (Ensheathed Bacteria)*

B. Cells flexuous, not rigid.

1. Cells elongate. Motility, by creeping on substrate.

*Order IV. Myzobacteriales (Mold-like Bacteria)*

2. Cells spiral. Motility, free swimming by flexion of cells.

*Order V. Spirochaetales (Spiral, Flexible Bacteria)*

*Supplements: Groups whose relationships are uncertain.*

1. Obligate intracellular parasites or dependent directly on living cells
  - a. Not ultramicroscopic and only rarely filtrable. More than  $0.1 \mu$  in diameter

*Group I. Order Rickettsiales*

- aa. Usually ultramicroscopic and filtrable. Except for certain pox viruses of animals and a few plant viruses, less than  $0.1 \mu$  in diameter.

*Group II. Order Virales*

2. Grow in cell-free culture media with the development of polymorphic structures including rings, globules, filaments, and minute reproductive bodies (less than  $0.3 \mu$  in diameter).

*Group III. Family Borrelomycetaceae*

The classification of the Eubacteriineae proposed in the sixth edition of Bergey's Manual, together with a brief description of the constituent tribes and genera, is presented in the Appendix.

In the seventh edition of Bergey's Manual a different classification is advanced. First of all it is proposed that the bacteria, the viruses, and the blue-green algae be placed in a new division (phylum) of the plant kingdom, this division (I) to be known as *Protophyta* (primitive plants). The yeasts, molds, and the higher algae remain in the *Thallophyta* which becomes division II of the plant kingdom. It is proposed to divide the *Protophyta* into three classes: the *Schizophyceae* or blue-green algae, the *Schizomycetes* or bacteria, and the *Microtobiotes* or smallest life, i.e., the rickettsiae and the viruses. In this new classification at least 200 genera of bacteria are recognized in 47 families placed in 10 orders.

Division into orders in the new system is primarily on the basis of motility, the first three orders being composed of polarly flagellated bacteria or species closely related to the polar flagellates. The next three orders contain peritrichously flagellated or related species; the following three orders contain the bacteria that glide, creep, or are flexuous in their movements when they are motile; the tenth order consists of apparently nonmotile parasites. The proposed classification (based on an outline supplied through the courtesy of Dr. H. J. Conn and a general review by Dr. R. S. Breed (1956) and reproduced in part here through the courtesy of the trustees of Bergey's Manual and The Williams and Wilkins Company), together with brief descriptive terms applied by the author, is as follows:

Division I. *Protophyta*—primitive plants.

Class I. *Schizophyceae*—blue-green algae.

Class II. *Schizomycetes*—fission fungi, the bacteria.

Gram-negative, polarly flagellated (or related non-motile) rods and curved forms (orders I to III).

Order I. *Pseudomonadales*—polar flagellate, true bacteria.

Suborder I. *Rhodobacteriineae*—pigmented, photosynthetic bacteria.

Family I. *Thiorhodaceae*—purple sulfur bacteria.

Family II. *Athiorhodaceae*—purple and brown nonsulfur bacteria.

Family III. *Chlorobacteriaceae*—green sulfur bacteria.

Suborder II. *Pseudomonadineae*—nonphotosynthetic pseudomonads.

Family I. *Nitrobacteraceae*—nitrifying bacteria.

Family II. *Methanomondaceae*—methane, carbon monoxide, or hydrogen oxidizers.

Family III. *Thiobacteriaceae*—sulfur oxidizers.

Family IV. *Pseudomonadaceae*—ordinary heterotrophic pseudomonads.

Family V. *Caulobacteraceae*—stalked bacteria.

Family VI. *Siderocapsaceae*—capsules encrusted with iron.

Family VII. *Spirillaceae*—comma-shaped and spiral forms.

Order II. *Chlamydo bacterales*—filamentous, colorless alga-like species.

Family I. *Chlamydo bacteraceae*—free filaments, motile swarm cells.

Family II. *Peloplocaceae*—folded filaments.

Family III. *Crenotrichaceae*—attached, differentiated filaments, form conidia.

Order III. *Hyphomicrobiales*—spherical to pear-shaped cells, often reproducing by budding at end of stalks.

Family I. *Hyphomicrobiaceae*—buds formed on fine stalks.

Family II. *Pasteuriaceae*—multiply by budding or by longitudinal fission.

Peritrichous and related types of true bacteria (Order IV).

Order IV. *Eubacteriales*—true bacteria (along with Order I).

Family I. *Azotobacteraceae*—gram-negative, nitrogen-fixing (free) bacteria.

Family II. *Rhizobiaceae*—gram-negative, symbiotic nitrogen-fixing or violet-pigmented bacteria.

Family III. *Achromobacteraceae*—gram-negative cells with little fermentative ability.



Family IV. *Enterobacteriaceae*—gram-negative, active fermenters, often found in enteric tract.

Tribe I. *Escherichieae*—relatively nonpathogenic.

Tribe II. *Erwinieae*—plant pathogens.

Tribe III. *Serratieae*—red pigmented.

Tribe IV. *Proteeae*—lactose not fermented, urea hydrolyzed.

Tribe V. *Salmonelleae*—generally pathogenic species.

Family V. *Brucellaceae*—small, gram-negative, obligate parasites.

Family VI. *Bacteroidaceae*—gram-negative, anaerobic rods.

Family VII. *Micrococcaceae*—gram-positive, non-chain-forming cocci.

Family VIII. *Neisseriaceae*—gram-negative cocci.

Family IX. *Brevibacteriaceae*—gram-positive, asporogenous rods, form little acid from sugars.

Family X. *Lactobacillaceae*—gram-positive cells, generally form lactic acid

Tribe I. *Streptococcaceae*—pairs and chains of cocci.

Tribe II. *Lactobacilleae*—rods occurring singly, in pairs, or in chains.

Family XI. *Propionibacteriaceae*—gram-positive, propionic or butyric acid-forming rods.

Family XII. *Corynebacteriaceae*—gram-positive, pleomorphic rods, weak fermenters.

Family XIII. *Bacillaceae*—generally gram-positive, aerobic or anaerobic, spore-forming rods.

Generally nonmotile, filamentous, mold- or alga-like bacteria (Orders V-VIII)

Order V. *Actinomycetales*—slender, often branching, mold-like cells which may form spores.

Family I. *Mycobacteriaceae*—acid-fast, asporogenous rods.

Family II. *Actinomycetaceae*—mycelial growth, divide by segmentation.

Family III. *Streptomycetaceae*—conidia formed on sporophores.

Family IV. *Actinoplanaceae*—sporangiospores, some flagellated.

Order VI. *Caryophanales*—large, filamentous, segmented cells.

Family I. *Caryophanaceae*—coenocytic, tubular cells, no spores.

Family II. *Oscillospiraceae*—large, partitioned, motile, sporogenous parasites.

Family III. *Arthromitaceae*—large, apparently septate, nonmotile, sporogenous parasites.

Groups of cells which often creep or glide over moist surfaces (Orders VII-VIII)

Order VII. *Beggiatoales*—filamentous, alga-like cells; some filaments may be motile.

Family I. *Beggiatoaceae*—oxidize sulfide, deposit *S* granules.

Family II. *Vitreoscillaceae*—organotrophic rather than *S* oxidizers.

Family III. *Leucothrichaceae*—organotrophic, tapering threads from lobiflats—only gonidia motile; gonidia form rosettes.

Family IV. *Achromotiaceae*—spherical to ovoid cells, may deposit *S* and/or  $\text{CaCO}_3$  granules.

Order VIII. *Myxobacterales*—slime-forming, slender, flexible rods, creeping motility.

Family I. *Cytophagaceae*—no fruiting bodies (cysts) or spores.

Family II. *Archaeomicrobium*—fruiting bodies of no definite shape, elongate spores (microcysts).

Family III. *Sorangium*—cysts usually angular, cells usually thick and short with blunt, rounded ends.

Family IV. *Polysporium*—cysts usually rounded, cells long and thin with pointed ends.

Family V. *Micrococci*—microcysts spherical to ellipsoidal, fruiting bodies formed except in genus *Sporocytophaga*.

Slender, flexuous cells, motility serpentine or by spinning (Order IX).

Order IX. *Spirochaetales*—spiral cells, some resemblance to protozoa.

Family I. *Spirochaetaceae*—large, mostly free-living forms.

Family II. *Treponemataceae*—small, generally parasitic cells.

Small, fragile, pleomorphic, nonmotile, often filtrable cells (Order X).

Order X. *Mycoplasmales*—the pleuropneumonia group.

Family I. *Mycoplasmataceae*—as for the order.

Obligate parasites, microscopic to ultramicroscopic in size (Class III).

Class III. *Microtobiotes*—smallest life.

Order I. *Rickettsiales*—small, intracellular parasites often associated with arthropods.

Family I. *Rickettsiaceae*—do not occur in erythrocytes.

Tribe I. *Rickettsiae*—cause human rickettsioses.

Tribe II. *Ehrlichiae*—cause animal rickettsioses.

Tribe III. *Wolbachiae*—arthropod, not vertebrate hosts.

Family II. *Chlamydiaceae*—found in tissues, not transmitted by arthropod vectors.

Family III. *Bartonellaceae*—occur in or on erythrocytes.

Family IV. *Anaplasmataceae*—parasites in erythrocytes of lower animals.

Order II. *Virales*—the filtrable viruses, nomenclature and classification uncertain

The descriptions of the various orders and families presented above are incomplete, and exceptions are encountered. Minor changes may be introduced before the seventh edition of Bergey's Manual is published and, therefore, the scheme should be considered as a tentative rather than a final one. Some changes in spelling are introduced in the new system, e.g., according to international rules for classification and spelling of terms the *i* in the former order and family names derived from species names containing bacter (but not bacterium) is omitted after the *r* before *-ales* and *-aceae*. The student is referred for details to "Bergey's Manual of Determinative Bacteriology," seventh edition. This new classification represents to a considerable extent reshuffling of genera, families, and orders into new arrangements, or in some instances changes of rank of taxa; but a new class and three new orders were created.

## THE PSEUDOMONADALES

Order I, the Pseudomonadales, and order IV, the Eubacteriales, consist primarily of those bacteria classified in the sixth edition of Bergey's

Manual as Eubacteriales. The Pseudomonadales, which includes all of the polar-flagellate and related types of bacteria, is subdivided into two suborders on the basis of energy source for the organisms—the photosynthetic species being classified as suborder I, the Rhodobacteriineae, and the chemosynthetic species as suborder II, the Pseudomonadineae.

**Rhodobacteriineae.** This suborder of the Pseudomonadales is composed of three families, the Thiiorhodaceae, Athiorhodaceae, and Chlorobacteriaceae, characterized by their ability to use light as a source of energy. Morphologically, these photosynthetic bacteria closely resemble other species of the true bacteria. They are gram-negative, asporogenous, spherical, rod- or curved-shaped cells ranging in size from 1 to more than 20  $\mu$ . These bacteria are characteristically pigmented red, purple, brown, or green, the color being due to the possession of a bacterial chlorophyll and usually one or more carotenoid pigments. The chlorophyll is located in minute bodies called grana. All species grow under anaerobic conditions in the light, while a few are also capable of growth under aerobic conditions in the dark. The photosynthetic bacteria may be characterized, to a great extent on the basis of the careful physiological and morphological studies of van Niel, in a general way as follows:

**Thiiorhodaceae.** These bacteria, which are widely distributed in sulfide-bearing waters, long constituted a puzzle in that while light was necessary for growth, oxygen was not liberated as a waste product as in the photosynthetic activity of the green plants. Current theories postulate that water is split under the influence of light and chlorophyll into a hydrogen and a hydroxyl radical, the hydrogen being utilized for the reduction of carbon dioxide fixed by the cell. The hydroxyl radical is decomposed by the green plants with the formation of water and oxygen. The photosynthetic bacteria are unable to carry out this reaction and instead reduce the hydroxyl radical with hydrogen obtained from compounds of sulfur, from organic matter, or with hydrogen gas. Water is formed as a result of the reduction while the hydrogen donor is oxidized, e.g., hydrogen sulfide to sulfur or sulfur to sulfate. The Thiiorhodaceae are the purple-sulfur bacteria—bacteria containing bacterial chlorophyll and various red and yellow carotenoid pigments. Hydrogen sulfide generally is utilized as the hydroxyl-reducing agent, sulfur granules frequently being deposited within the cells. These bacteria are autotrophic in their nutritional requirements, although many species can employ simple organic compounds instead of hydrogen sulfide or other reduced compounds of sulfur, as the hydrogen source.

**Athiorhodaceae.** This family is composed of the purple and brown, non-sulfur-containing, photosynthetic bacteria. These bacteria rather closely resemble those species constituting the Thiiorhodaceae, the main physiological differences being that organic matter is the preferred source



of hydrogen (some species may use thiosulfate or hydrogen gas) for the reduction of the hydroxyl radical and that organic growth factors are required. The Athiorhodaceae are, therefore, more heterotrophic in their metabolism than the Thiorhodaceae. In addition they exhibit a greater tendency to grow as heterotrophs in the dark under aerobic conditions. Morphologically, all known species quite closely resemble the true, non-photosynthetic bacteria, particularly of the family Pseudomonadaceae, although in older cultures, involution forms do resemble members of the genera *Corynebacterium* and *Mycobacterium*.

*Chlorobacteriaceae*. These, as the name implies, are the green bacteria, their color being due to the possession of a green bacteriochlorophyll somewhat different in composition from the bacteriochlorophyll of the other two families of photosynthetic bacteria. They are all small bacteria capable of growth in the presence of light under anaerobic conditions in an inorganic medium. Hydrogen sulfide is commonly employed as the source of hydrogen, being oxidized to sulfur, which is generally deposited outside the cells.

*Pseudomonadineae*. This suborder of the Pseudomonadales is composed of a metabolically and morphologically heterogeneous group of organisms characterized by polar flagellation and chemosynthetic (energy obtained from oxidations) mode of life. As constituted in the seventh edition of Bergey's Manual the suborder comprises bacteria formerly classified in two different orders and in different families as well. The constituent families, established on the basis of both physiological and morphological characteristics, can be described briefly as follows:

*Nitrobacteraceae*. These are autotrophic bacteria capable of growing in an inorganic medium with carbon dioxide as the sole source of carbon, energy being obtained from the oxidation of ammonia to nitrite or of nitrite to nitrate. *Nitrosomonas* (ammonia oxidizer) and *Nitrobacter* (nitrite oxidizer) are representative genera. The constituent genera are established on the basis of which of the two compounds is oxidized and on morphological grounds. These bacteria are found in soil and in water and are of extreme importance in the economy of nature, converting ammonia into nitrates, which are essential for plant growth. Other autotrophic species formerly were included in this family but now are classified separately.

*Methanomnadaceae*. This family is composed of pseudomonads on the border line between the autotrophs and the heterotrophs, many species being capable of growth on truly heterotrophic media. The constituent genera are established on the basis of their most characteristic energy source—*Methanomonas* oxidizing methane, *Hydrogenomonas* oxidizing hydrogen gas, and *Carboxydomonas* growing at the expense of carbon monoxide, which is highly lethal to most forms of life.



*Thiobacteriaceae*. This family is composed of those autotrophic bacteria which oxidize sulfur, thiosulfate, sulfide, or other reduced compounds of sulfur, obtaining energy for maintenance and growth from these oxidations, in contrast to the photosynthetic bacteria which utilize such compounds as a source of hydrogen rather than of energy. These sulfur-oxidizing bacteria, such as *Thiobacterium* and *Macromonas*, are, like the nitrifying bacteria, of extreme importance in the maintenance of soil fertility. Sulfur, as such, or sulfide from the decomposition of cellular material are the main supplies of sulfur for green plants and must be oxidized by the sulfur bacteria to sulfate to render the element available to the plant. One species, *Thiobacillus thiooxidans* is characterized by its ability to produce sulfuric acid (sulfate) and grow in highly acidic solutions (pH 1.0 or less). *Thiobacillus denitrificans* grows anaerobically, oxidizing sulfur compounds at the expense of nitrates.

*Pseudomonadaceae*. Twelve genera are proposed for this family of heterotrophic, polarly flagellated rods. The first three, *Pseudomonas*, *Xanthomonas*, and *Acetobacter*, are the best known, many of the other genera being of less frequent occurrence and characterized either by odd metabolic activities such as the oxidation of protamine, alginic acid, or aromatic substances such as phenol, or by their ability to produce light or to grow in highly concentrated salt solutions.

Species of *Pseudomonas* are most commonly found in soil and water, although a few are animal pathogens and more are plant pathogens. One hundred and fifty or more species have been described, differing from each other primarily in various physiological characteristics. Many species produce bluish-green or yellowish-green pigments which are water soluble and diffuse through the medium. Species of the second genus, *Xanthomonas*, differ in that they produce a yellow, water-insoluble pigment and for the most part are plant pathogens causing necrosis in their hosts. The third major genus, *Acetobacter*, is characterized by aerobic fermentation, i.e., the inability of its constituent species to carry out complete oxidation of most of the substrate to carbon dioxide and water. Instead, a major portion of the substrate is partially oxidized, ethyl alcohol for example being oxidized to acetic acid by the *Acetobacter* employed in the preparation of vinegar.

*Caulobacteraceae*. This group of bacteria formerly was given the rank of a suborder but is now classified as a family of Pseudomonadales. The Caulobacteraceae, as the name implies, are the bacteria which characteristically grow upon stalks attached to a substrate. The cells are generally single and kidney-shaped or spherical, the stalk being secreted from one side of the cell and the base of the stalk held to a surface by means of a little holdfast. In some species the stalk is very short or absent and the cells are frequently attached to a surface in a zoogloecic

mass. The stalk is composed of ferric hydroxide in the genus *Gallionella* and of organic matter in the genera *Caulobacter*, *Siderophagus*, and *Nesokia*. Most species of the Caulobacteraceae are saprophytes living in an aquatic environment. They have been studied only to a limited extent since many of the species cannot be cultivated readily, if at all, in the laboratory in pure culture.

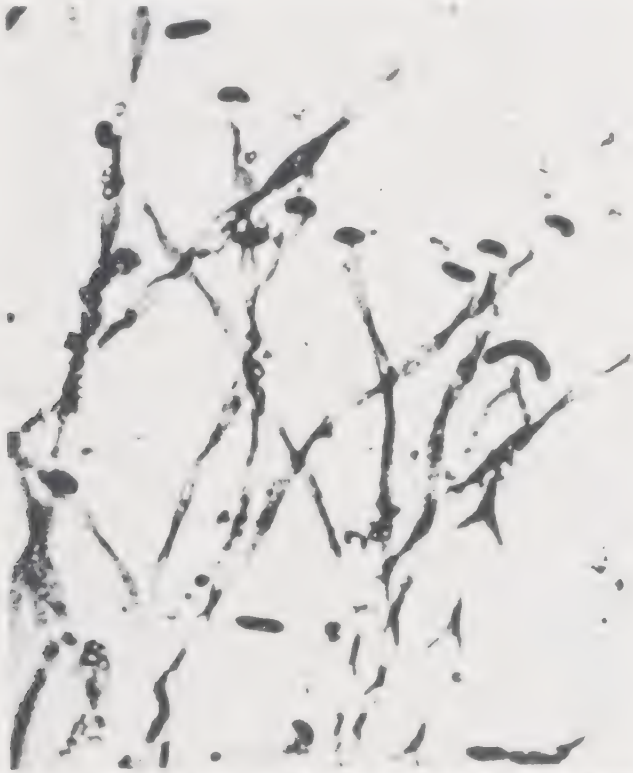


FIG. 6-1. Photomicrograph of *Gallionella ferruginea*, an iron bacterium. (From "Die Eisenbakterien" by H. Molisch, Jena, 1910; print by courtesy of R. L. Starkey.)

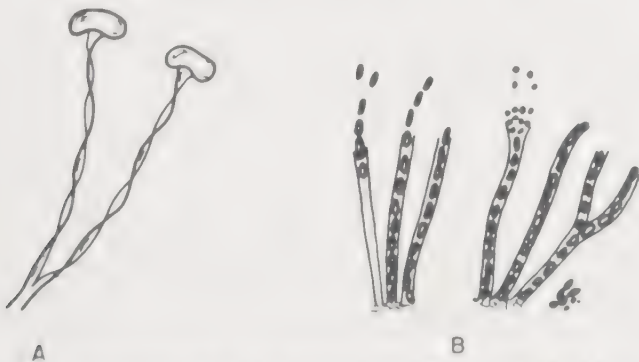


FIG. 6-2. Schematic illustration of (A) stalked and (B) ensheathed bacteria, depicting false branching in the latter.

The most generally known species in this family is *Gallionella ferruginea*, a small kidney-shaped bacterium, 0.5 by 1.2  $\mu$ , which secretes ferric hydroxide from the concave portion of the cell to form band-like stalks. A rotatory motion of the cells causes a spiral twisting of the stalks as illustrated in Figs. 6-1 and 6-2. This species, and similar bacteria, are found in cool, iron-containing waters and are frequently a nuisance in water distribution systems, the deposit of iron in time decreasing the rate of flow of the water. The cell masses, on breaking free from the supporting surfaces, discolor the water and may become quite a nuisance, although harmless as far as disease is concerned.

*Sulzerocapsaceae*. This family is not well known and consists to a great extent of spherical or ovoid cells embedded in gelatinous masses, the capsules becoming encrusted with ferric hydroxide. Motile stages, if any, are unknown, but the cells resemble other species of Pseudomonadales and are included in this order. They have not been cultivated on artificial media and are found, for the most part, growing attached to the surfaces of leaves and other parts of water plants. There is some resemblance to the Caulobacteraceae, but the significance of the iron deposit is unknown.

*Spirillaceae*. This, the seventh family of the Pseudomonadineae, is comprised of curved, polarly flagellated bacteria of widely different physiological types. Ten genera are recognized on the basis of physiological and morphological characteristics. They are usually found in soil or water, although a few species, particularly in the genus *Vibrio*, are pathogenic for man or other animals. *Vibrio comma*, the bacillus of Asiatic cholera, is the most notorious pathogen in the order Pseudomonadales.

### THE CHLAMYDOBACTERALES

The name Chlamydobacterales suggests that these are sheathed bacteria, and most species are commonly enclosed in a sheath, which may be inorganic or organic in character. They are colorless, filamentous bacteria which in structure rather closely resemble certain of the algae. They are aquatic in habitat, some of them being attached to objects in the water while others are free-floating. This order is divided into three families—Chlamydobacteraceae, Peloplocaceae, and Crenotrichaceae—primarily on the basis of morphology. The families Chlamydobacteraceae and Crenotrichaceae do not contain sulfur granules and may exhibit false branching, the former family usually existing as free filaments, while species of the latter family are generally attached to an object. Motile swarm cells can be formed by members of the first family, nonmotile conidia by the latter. The family Peloplocaceae consists of two genera,

*Peloplaea* and *Pelonema*, found in mud. The organisms are filamentous, tend to occur in stiff bundles, and have a tendency to bend or fold upon themselves. Relatively little information is available concerning this family. The Beggiatoaceae were formerly included in the order Chlamydobacterales, but they have been shifted to a new order, called the Beggiatoales.

*Chlamydobacteraceae*. This family consists of three genera—*Sphaerotilus*, *Leptothrix* and *Toxothrix*—species of which may or may not show false branching or deposition of ferric hydroxide in the sheaths. In most species the sheath consists of polysaccharides or other gummy material which may be impregnated with ferric hydroxide; in other species the sheath is composed entirely of ferric hydroxide. At times a cell can become detached from others in the sheath and finally push out through it, continuing to grow and to form a new sheath. This (see Fig. 6-2) is responsible for the appearance of branching in cultures of these bacteria. When the sheaths become very thick, the cells or filaments slip out, leaving the empty sheaths behind. These empty sheaths can be observed at times when one examines deposits from iron springs under the microscope. The iron-depositing species of this family can interfere with the flow of water through pipes as previously mentioned for the *Gallionella*. It was long believed that the iron-depositing bacteria obtained part or all of the



FIG. 6-3. Photomicrograph of ensheathed iron bacteria.



energy required for growth from the oxidation of reduced iron compounds. At least one species has been cultivated in an organic medium with only a trace of iron present, and it has not been cultivated in an iron-containing, organic-free medium. Other species have not been obtained in pure culture, and the role of iron in their respiration remains obscure. There is some evidence suggesting that deposits of bog iron ore are due to the action of the iron bacteria.

The genus *Sphaerotilus* is composed of species which form sheaths entirely organic in character. The type species, *Sphaerotilus natans*, forms a slimy sheath, 2 to 3  $\mu$  in diameter, within which multiplication occurs by the formation of conidia. The conidia swarm out of the open end of the sheath; they may swim around for some time and finally attach themselves to some object and develop into slender filaments. The base, or holdfast, of the filament is mucilaginous in character. The cylindrical cells within the filament multiply and are arranged end to end in the sheath, which they secrete about them. Occasionally one may be displaced in the sheath and, as it continues to multiply, gives rise to a new filament passing from the original one and therefore to a branched appearance, or false branching. The cells themselves do not branch. This organism may become quite a nuisance in sewage-disposal plants.

*Crenotrichaceae*. Since so little information is available concerning the Peloplocaceae, we will skip consideration of it and consider next the third family, the Crenotrichaceae. This family is composed of three genera, *Crenothrix*, *Pragmadiothrix*, and *Clonothrix*, of nonbranching, filamentous cells which are spherical to cylindrical in shape and which divide in three planes to form spherical, nonmotile conidia in the tips of the sheath tubes, the latter expanding from the base toward the tip. The conidia may become attached to an old sheath, giving rise to new filaments and thus simulating false branching. The genus *Clonothrix* is composed of rather large, cylindrical cells which deposit iron or manganese in the organic sheath. The sheath narrows towards the tip.

### THE HYPHOMICROBIALES

This is a new order of bacteria which can reproduce by buds formed at the end of long stalks that grow out from the cells (see Fig. 6-4). The Hyphomicrobiales are divided into two families—the Hyphomicrobiaceae, in which the buds are formed on slender stalks, and the Pasteuriaceae, which multiply by budding or by longitudinal fission. The relationship of this family to other families of bacteria is not known. Two genera are recognized in both families: *Hyphomicrobium* and *Rhodimicrobium* (photosynthetic species) in the former, *Pasteuria* and *Blastocaulis* in the latter.

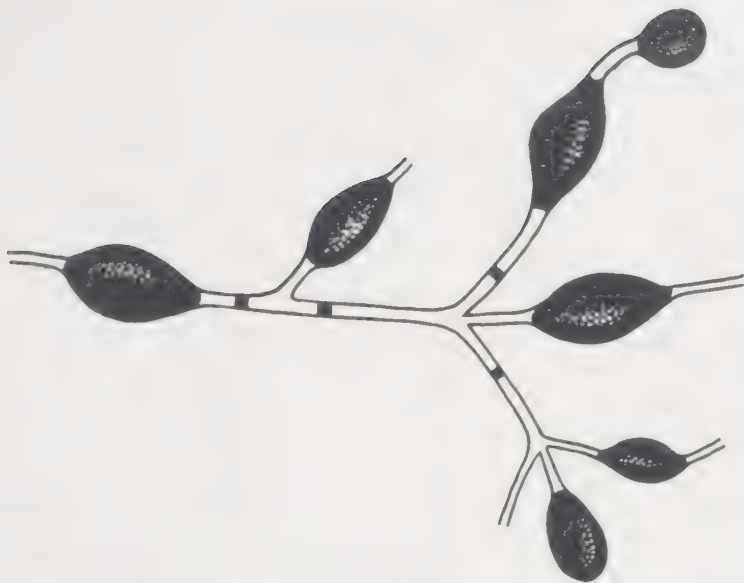


FIG. 6-4. Schematic illustration of *Rhodobacterium ramielli*. [Redrawn from Duchow and Douglas, *Journal of Bacteriology*, **58**, 411 (1949).]

### THE EUBACTERIALES

The bacteria in this order, along with those in the first order, constitute the true bacteria in the narrower use of the term "bacteria." The Eubacteriales are composed of peritrichously flagellated bacteria and related nonmotile species. The order is divided into thirteen families, indicated in the outline for the classification proposed in the seventh edition of Bergey's Manual. Many of the order's members will be considered in more detail as we consider the bacteria and their activities; the Eubacteriales will not be described here.

### THE ACTINOMYCETALES

The order Actinomycetales constitutes somewhat of a transition group between the true bacteria and the higher fungi in that most species form elongated cells which exhibit a marked tendency to branch in a manner analogous to the molds. The filaments, however, are thinner than those of the molds and generally are less than one micron in diameter. Four families are recognized on the basis of general morphological considerations: the Mycobacteriaceae, Actinomycetaceae, Streptomycetaceae, and Actinoplanaceae. These may be characterized as follows:

*Mycobacteriaceae*. Members of this family have more in common with the true bacteria than do the other families of the order, exhibiting resemblances to the Corynebacteriaceae in particular. The major genus in

this family, *Mycobacterium*, is composed of slender, nonmotile, gram-positive rods which may show a slight tendency to branch in old cultures; the other genus, *Mycococcus*, is spherical in form. All species are acid fast, a staining characteristic exhibited to some extent by certain species of the Actinomycetaceae, particularly in the genus *Nocardia*. Spores are not formed by members of this family. The tubercle bacillus is the best-known member of the family. It tends to multiply much more slowly than do other members, several weeks' incubation often being required before good growth is evident from light inocula.

*Actinomycetaceae*. Members of this family constitute a transition group between the Mycobacteriaceae and the Streptomycetaceae; like the latter family, the Actinomycetaceae form branched filaments of mycelium, but these filaments fragment in older cultures into short elements which look like true bacteria or like tubercle bacilli. These fragmentation forms are similar to the oïdia formed by some species of the higher fungi and are called oïdia or arthrospores. Chlamydospores may also be formed in older cultures.

The family Actinomycetaceae is divided into two genera, the aerobic *Nocardia* and the microaerophilic or anaerobic *Actinomyces*. Many members of the former genus are able to oxidize paraffin or phenols as a source of energy. The *Actinomyces* species are parasitic. *A. bovis* causing

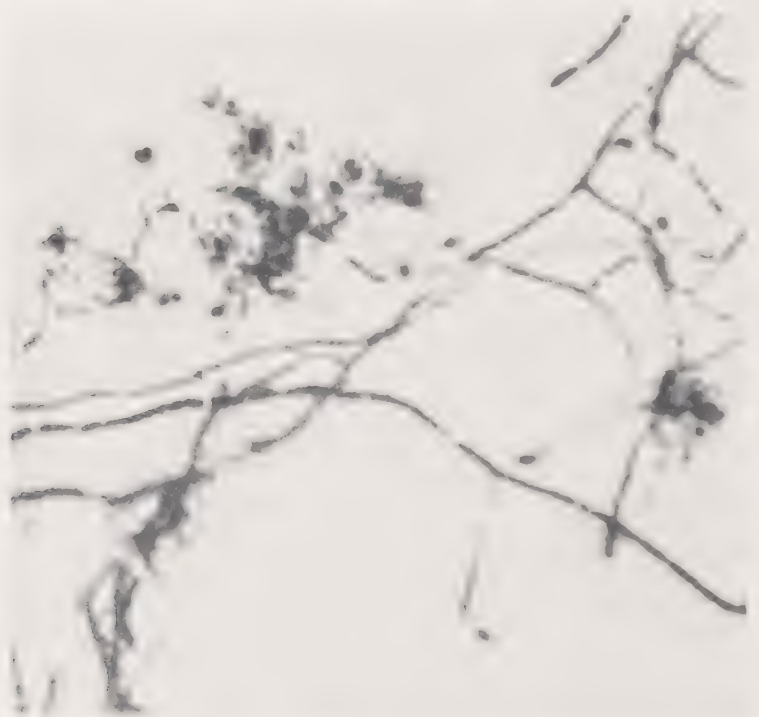


FIG. 6-5. Photomicrograph of a species of *Nocardia*

lumpy jaw in cattle and *A. israeli* an actinomycosis in man. *Nocardia multarae* causes an infection known as Madura foot in man.

*Streptomycetaceae*. The third family of the Actinomycetales most closely resembles the molds, producing a tangled mycelial mass with conidia borne at the end of hyphae. The filaments, like those of the Actinomycetaceae, are much thinner than in the true molds and are generally not greater than  $1.5\ \mu$  in diameter. They never fragment, as do the Actinomycetaceae, into coccoidal or bacillary forms. The members of this family are primarily soil forms, being of great value in the maintenance of soil fertility and, in particular, aiding in the breakdown of complex organic matter.

This family is divided into three genera; the first two established on the basis of spore formation, the third, *Thermoactinomyces*, on the basis of heat resistance. The conidia are borne singly at the ends of short conidiophores in the genus *Micromonospora*, in chains at the ends of aerial conidiophores in the *Streptomyces*. More than seventy species are recognized in the latter genus, particularly on the basis of biochemical activities and pigment formation. A large number of species produce antibiotic agents, *Streptomyces griseus*, e.g., forming streptomycin.

*Actinoplanaceae*. Relatively little information is available considering this family of the Actinomycetales. Individual species appear to form sporangiospores rather than conidiospores, and in some species, at least, the spores are flagellated.

### THE CARYOPHANALES

This order is composed of filamentous or rod-shaped bacteria, often quite large (5 microns wide by 20-50 microns long) and quite complex structurally. The central bodies frequently referred to have been shown to be analogous to the nuclei of other bacteria, while the ring-like bodies evident in many cells are transverse septa. These septa can be observed in unstained cells and, together with the outer walls, in some lysed preparations. The Caryophanales are divided into three families—Caryoplanaceae, Oscillospiraceae, and Arthronitaceae—on the basis of morphology (see outline). Little is known as yet of the general activity of this group in nature.

### THE BEGGIATOALES

Winogradsky around 1887 demonstrated conclusively that the large filamentous bacterium *Beggiatoa alba* deposited small granules of sulfur within the cell when grown in the presence of hydrogen sulfide. Upon depletion of the sulfide the intracellular sulfur was oxidized to sulfate.



These autotrophic organisms are found in waters which contain hydrogen sulfide and often give rise to extensive white coatings of fine interlacing filaments deposited upon the bottom of the stream. The filaments are composed of chains of cells, and in this respect *Beggiatoa* appears to be related to the Chlamydobacterales. Motility, when present, does not appear to be the result of flagellar activity but instead is more of a gliding, the extremities of the filaments often waving back and forth. In this motion and in its cytology *Beggiatoa* resembles some of the blue-green algae in the family Oscillatoriaceae. Primarily on the basis of motility the *Beggiatoa* and related bacteria are placed in a new order, the Beggiatoales, rather than in the Chlamydobacterales, in the seventh edition of *Bergey's Manual*.

Four families are proposed in the new classification: Beggiatoaceae, Vitreoscillaceae, Leucotrichaceae, and Achromotiaceae. These were described briefly in the outline. The family Achromotiaceae is included in this order although the cells occur singly. Motility, however, is jerky or rotational in character, and no organs of locomotion are known. The Vitreoscillaceae, described by Pringsheim in 1951, are closely related morphologically to the Beggiatoaceae but obtain energy from the oxidation of organic matter rather than of sulfide or intracellularly deposited sulfur.

### THE MYXOBACTERIALES

These are commonly called the slime bacteria, because the vegetative cells form and multiply within a slimy matrix to produce thin, irregularly



FIG. 6-6. Photomicrographs of colonies and cells of myxobacteria. (From Nowak, "Documenta Microbiologica," Gustav Fischer, Jena, 1927.)

1. *Myxobacter subaerius*. The larger globules are fruiting bodies.
2. (A) *Myxococcus stipitatus*, fruiting bodies.  
(B) *Chondromyces auranticus*, fruiting bodies.
3. Colony of *Sorangium compositum*.

shaped, spreading colonies, or pseudoplasmodia, often spoken of as the swarm stage. The actively growing vegetative cells are relatively long, slender, flexible, nonflagellated rods. These rods are motile, but motion is due to some mechanism other than propulsion by flagella. The cells frequently are arranged in groups of 2 to 12 or more lying parallel to each other, the group moving as a unit on a thin layer of slime which the cells excrete, motion being away from the center of the colony. It has been suggested that this creeping or crawling motion is due to asymmetrical slime production, the greatest production at one end pushing the unit along, possibly with some aid from the individual cells comprising the unit.

Most of the genera of the Myxobacterales are also characterized by the formation of fruiting bodies, or cysts. When the pseudoplasmodium approaches maturity, the cells tend to collect in a mass or masses, which in many instances are elevated above the substratum on a mucilaginous base which hardens to some extent. The cells pass into a resting stage, becoming shortened and thickened and frequently developing a relatively thick, highly refractile wall. These are known as microcysts, or spores. In some species the fruiting bodies are simply finger-like projections containing microcysts; in other species the fruiting bodies show a greater degree of differentiation, the spores being enclosed within large cysts surrounded by a relatively firm membrane. A considerable degree of communal activity may be involved in the formation of fruiting bodies, particularly in those species in which branching fruiting bodies are produced with vegetative cells being used up in the process. The fruiting bodies are either sessile or stalked and are usually pigmented orange, red, yellow, or brown, though black or colorless bodies may be formed. The nature of the spores (microcysts) and of the fruiting bodies (cysts) varies with the different species and is employed as a basis for their classification into families. In one family, the Cytophagaceae, the fruiting stage is absent, but the other characteristics of the species in this family are those characteristic of the order as a whole. The families (the same in the sixth and seventh editions of Bergey's Manual) can be briefly characterized as follows:

- I. Cytophagaceae, neither definite fruiting bodies nor microcysts
- II. Archangiaceae, resting cells elongate, fruiting bodies not of definite shape but usually finger-like processes
- III. Sorangiaceae, resting cells elongate, cysts usually angular, vegetative cell thick and short with rounded ends
- IV. Polyangaceae, resting cells elongate, cysts usually rounded, vegetative cells long and thin with pointed ends
- V. Myxococcaceae, resting cells spherical to ellipsoidal, fruiting bodies vary with the species or are absent

Most of the known species are saprophytic and are commonly found on rotting wood or other complex vegetable matter or on dung. They frequently appear to live in close association with true bacteria and may be parasitic upon them. The family Cytophagaceae is best known. Species of the genus *Cytophaga*, like many members of this order, have the ability to decompose complex materials such as cellulose, and for this reason they are highly valuable in the maintenance of soil fertility, returning this material to the soil in usable form. At least one species is able to liquefy agar. Many species of this order have not been studied in pure culture. It may be that the members of this order form a transition group between the bacteria proper and the Myxomycetes, or slime molds.

### THE SPIROCHAETALES

This, the ninth order of the Schizomycetes, contains the relatively elongated, flexible, spiral bacteria which, like the Myxobacterales, differ from the other bacteria in that they do not possess rigid cell walls. Some of the simpler species in this order have characteristics similar to those of the genus *Spirillum* of the Eubacteriineae; others are approaching the Protozoa in structure or mode of life. Some species possess an axial filament that can be readily demonstrated, others a lateral crista, or ridge, and others transverse striations; otherwise there is no particular protoplasmic pattern. All forms are motile, motility being by serpentine motion or by spinning on the long axis, although recent electron micrographs suggest polar flagellation in some species. Multiplication is by transverse fission, and no sexual cycle is known. Most species are difficult to cultivate, or have not been cultivated, in the laboratory. They generally stain with difficulty with the ordinary bacteriological stains, special stains such as the Giemsa stain or impregnation with silver being required. They can be most readily observed in the dark field.

The order is divided (sixth and seventh edition of Bergey's Manual) into two families, the *Spirochaetaceae*, consisting of cells with definite protoplasmic structures and ranging from 30 to 500  $\mu$  in length, and the *Treponemataceae*, shorter spirals with no obvious protoplasmic structure. The Spirochaetaceae are primarily saprophytes commonly found in water polluted with sewage or other organic wastes, while the Treponemataceae are generally parasitic forms. The latter family consists of three genera: the *Borrelia*, which stain readily with aniline dyes and can (?) be cultivated in the laboratory; the *Treponema*, which are strict anaerobes, are stained with difficulty, and are strict parasites; and the *Leptospira*, which differ from the *Treponema* in being aerobes and have one or both ends characteristically hooked when cultivated in liquid media.

Certain species of *Borrelia* (e.g., *B. recurrentis*), often transmitted by



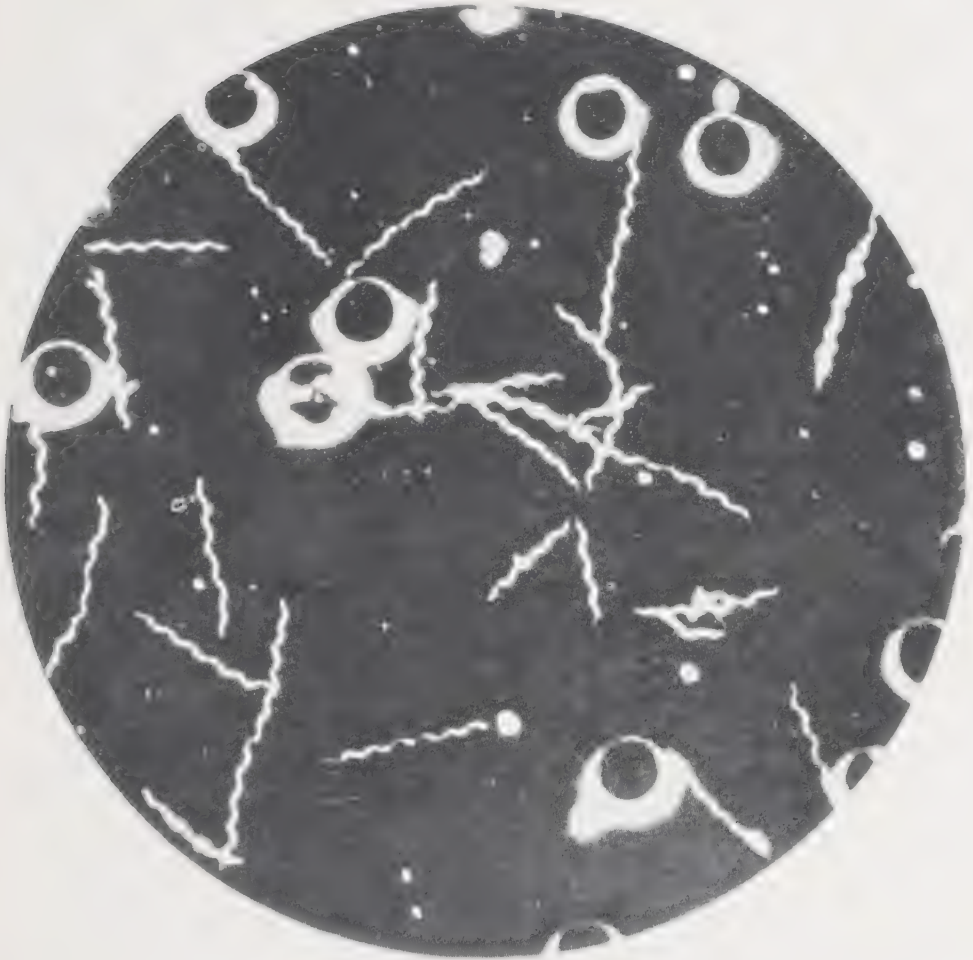


FIG. 6-7. Photomicrograph of spirochetes in a dark field. (Courtesy of the American Optical Company.)

insects, are the cause of relapsing fevers which resemble some of the trypanosome infections. Other pathogens in this family are *Treponema pallidum*, the agent of syphilis, and *Leptospira icterohaemorrhagiae*, the cause of infectious jaundice.

#### THE MYCOPLASMATALES

This, the tenth and last order of the Schizomycetes as defined in the seventh edition of Bergey's Manual, was considered the family Borrelomycetaceae in the previous edition. One family, Mycoplasmataceae, is now recognized and contains one genus, *Mycoplasma*. Since all the reasons behind this change and also the descriptions are not available at the time of writing the following discussion of the Mycoplasmatales



Borrelomycetaceae, or pleuropneumonia group is based upon earlier terminology and information.

In 1898 Nocard and Roux cultivated bacteria-like organisms from pleural exudate obtained from a cow ill with a disease now known as bovine pleuropneumonia. This organism (*Asterococcus mycoides*, *Mycoplasma mycoides*?) was shown to pass through filters which would retain ordinary bacteria, suggesting a possible relationship with the filtrable viruses. It grows barely, if at all, on ordinary media and more readily in the presence of up to 25 per cent of serum. In serum-enriched media the growth in several days gives rise to colonies barely visible to the naked eye. The cells, which are gram negative, do not stain readily with the ordinary aniline dyes commonly employed in the laboratory, and without special precautions they are generally destroyed in the preparation of the usual bacterial "smear."

In contrast to the bacteria, the pleuropneumonia organisms appear to be soft and fragile, lacking a rigid cell wall. In fluid media in particular, they are highly pleomorphic, giving rise to a wide variety of sizes and shapes—cocci, bacillary, curved, filamentous, and globular forms, ranging in size from about two-tenths of a micron to ten or more microns. The globular forms, or "large bodies," appear to be part of a reproductive cycle and to be produced by the swelling of bacillary or filamentous forms. These large bodies produce granules or filaments by internal segmentation or multiple germination, and the minute forms so produced are set free on autolysis of the large body. The minute reproductive bodies do not have the characteristics of spores. The development and subsequent autolysis of the large bodies tend to produce a roughened or granular appearance in the colonies of this group of organisms.

At least one organism in the pleuropneumonia group, *Streptobacillus moniliformis*, closely resembles the actinomyces in many respects but gives rise after several days' incubation to highly pleomorphic forms characteristic of the pleuropneumonia group. This was first believed to be the result of the growth of a second organism in association with the actinomyces-like form, and Klieneberger considered the pleomorphic form to be a pleuropneumonia type of organism, which she termed " $L_1$ ." Present evidence indicates that there is but one organism involved and that the actinomyces-like form and the  $L_1$  form represent different stages of growth of one organism characterized by a complex reproductive cycle. The  $L_1$  form is more resistant to heat and to penicillin than the actinomyces form, but in their fermentative and serological properties they are very similar or identical.

The majority of the members of the pleuropneumonia group are parasitic or pathogenic forms and are responsible for infections such as pleuropneumonia of cattle, related diseases in other animals, and agalactia, an

inflammatory infection of the mammary glands of lactating sheep and goats. However saprophytic species have been isolated from soil and from sewage.

Members of the pleuropneumonia group do not appear to be true bacteria in the sense of the definition and general descriptions which we have considered. They multiply by methods other than binary fission, require highly enriched media for growth, which is never luxuriant, produce extremely minute colonies, exhibit very marked variations in morphology, stain poorly with the ordinary bacteriological stains, are extremely fragile, and, at least in certain stages of growth, pass readily through bacteria-retaining filters. They more closely resemble the viruses in size, in filtrability, and pathogenicity, but they do grow to a limited extent on lifeless media and saprophytic forms do exist. Such considerations suggest that they belong in a group intermediate between the bacteria and the viruses. However other workers believe that the pleuropneumonia organisms are phases in the life cycle of bacteria and present evidence indicating that filtrable forms and large bodies are produced in cultures of typical bacteria including common species of *Proteus*, *Escherichia*, *Hemophilus*, *Neisseria*, and *Bacteroides*.

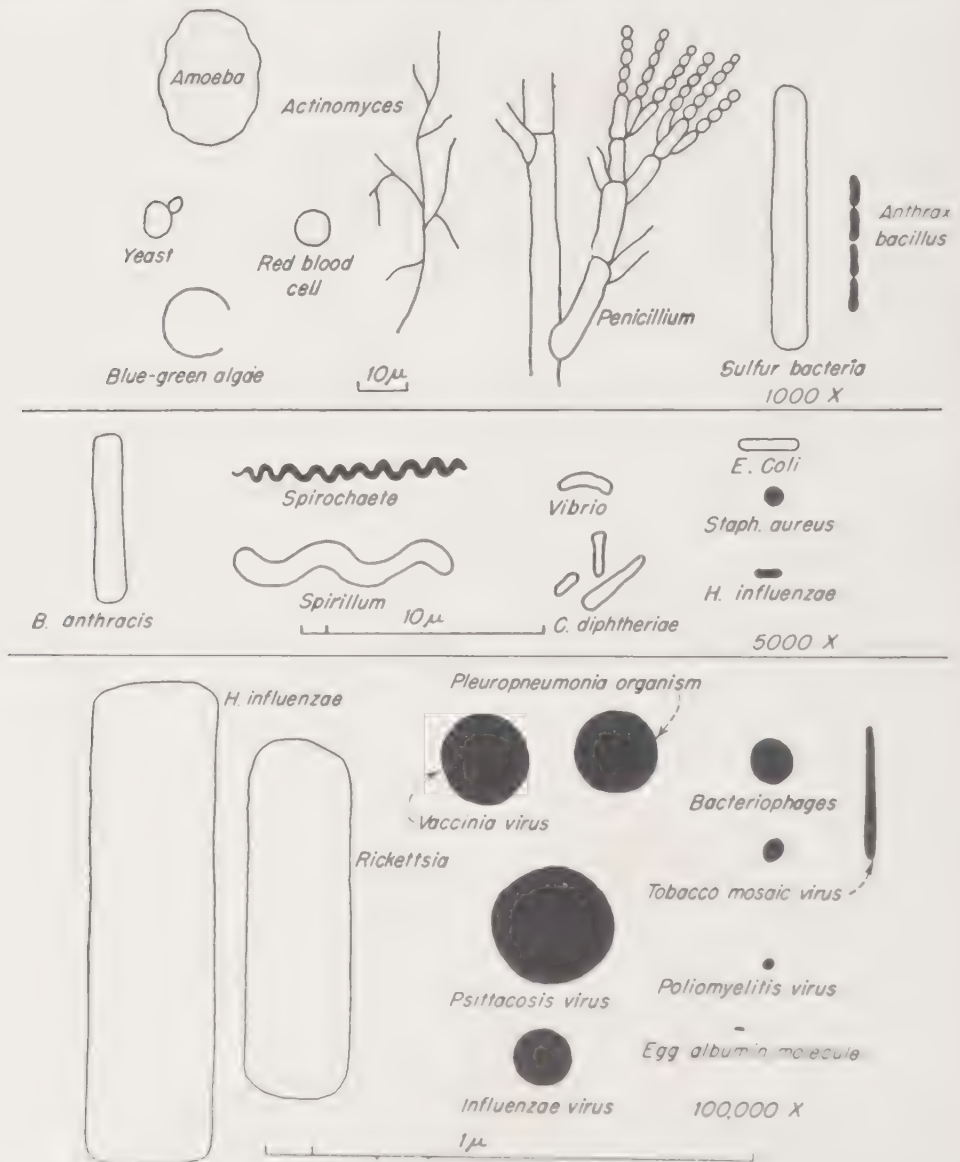
This sketchy consideration of the pleuropneumonia group of microorganisms suggests the heterogeneity of the bacteria and related forms and further illustrates the difficulties encountered in any classification of the various types of microorganisms and ultramicrobes into families, orders, and other groups and subgroups. Possibly, as Frobisher and others have suggested, there is a continuity of animate matter somewhat analogous to continuities such as wave motions encountered in inanimate matter, the continuity in the latter case being broken down into radio waves, X rays, visible light, heat, etc., on a rather arbitrary basis. The pleuropneumonia group does have characteristics exhibited at times by different bacteria, but for the present it might be preferable to consider it and the rickettsiae as transition groups between the bacteria and the viruses, much as the latter may be a transition group between animate and inanimate matter.

For more complete descriptions of the orders, families, genera, and individual species, for reference to the original literature on each, and for any changes that may be introduced, the student is again referred to the sixth and seventh editions of "Bergey's Manual of Determinative Bacteriology."

### THE RICKETTSIALES

In the outline of the classification proposed in the seventh edition of Bergey's Manual, a new class of Schizomyxetes, the Microtatobiotetes, is proposed. This class was defined as smallest life and is made up of two

orders, the Rickettsiales and the Virales. Nomenclature and classification of the latter taxon is in a state of flux, and no generally accepted scheme has been advanced. It appears that the Virales will be the subject of a separate volume. These organisms, the filtrable viruses, will be considered in the following chapter. The rickettsia appear to be closely related to the viruses, to some extent on the basis of size (see Fig. 6-8) but primarily on their inability, like that of the viruses, to multiply in lifeless media. The rickettsiae are found primarily in arthropods, in which they live without producing any apparent symptoms of disease.



6-8 A comparison of the relative sizes of different microorganisms and viruses



usually. On transmission to man or other animals, however, they are apt to cause infection. Morphologically the rickettsiae are more closely related to the bacteria than to the viruses and, therefore, will be considered here. The terminology employed, however, is that proposed in the sixth edition of Bergey's Manual because little information other than that given in the outline is available at the time of writing this chapter.

Ricketts in 1909 first recognized a minute organism, now known as *Rickettsia dermatroxenus* or preferably *Rickettsia rickettsii*, as the causative agent of Rocky Mountain spotted fever. In the following year he reported a similar organism as the cause of typhus fever (not to be confused with typhoid fever of bacterial origin), a finding definitely established in 1916 by da Rocha-Lima, who demonstrated the louse-borne nature of the disease. The latter named the causative agent of typhus fever *Rickettsia prowazekii* in honor of Ricketts and of Prowazek, both of whom lost their lives in the study of the disease.

These and other rickettsiae differ from the bacteria in their inability to grow in lifeless media, their poor staining properties, and their normal association with insect life, generally arthropods. They are more rigid and less pleomorphic than the pleuropneumonia group and differ from the viruses in their larger size and their inability (with one exception, *Coxiella burnetii*) to pass through the bacteria-retaining filters. In morphological characteristics they more closely resemble the bacteria than the viruses; in their parasitic mode of life the reverse holds true. Hence they may be considered for purposes of discussion as a transitional group between the bacteria and the viruses.

The Rickettsiales (see Fig. 24-10) are frequently pleomorphic organisms, 0.2 to 0.5  $\mu$  in diameter and up to 2  $\mu$  in length in the rod-shaped forms. They are spherical, rod-shaped, or irregularly shaped organisms and are gram negative in their staining characteristics. They can most readily be demonstrated in Giemsa or similarly stained preparations. These agents are more rigid than the pleuropneumonia group and, in contrast to the latter and the bacteria, grow only in living tissues (generally intracellularly) *in vivo* or *in vitro*. They are primarily parasitic upon arthropods, which act as vectors, but they can be transmitted by these hosts to man and other animals. In the sixth edition of Bergey's Manual they are divided into three families, which can be characterized as follows:

#### KEY TO THE FAMILIES OF THE ORDER RICKETTSIALES

Either intracellular parasites, or intimately associated with tissue cells. Generally transmitted by arthropod vectors

##### *Family I. Rickettsiaceae*

Facultative parasites found characteristically in or on the erythrocytes of vertebrates. May be transmitted by arthropod vectors



KEY TO THE FAMILIES OF THE ORDER RICKETTSIALES (*Continued*)*Family II. Bartonellaceae*

Intracellular parasites found in vertebrate tissues. Not transmitted by arthropod vectors

*Family III. Chlamydozoaceae*

The family Rickettsiaceae has been divided into three genera, the *Rickettsia*, *Coxiella*, and *Cowdria*. Since the latter two genera contain but one species each, and since these have been created primarily on the basis of filtrability of the second genus and on the basis of a tendency to a spherical shape in the third genus, there is considerable question as to the validity of such a classification. Therefore they will be considered as a unit for our purposes of discussion.

The rickettsiae tend to parasitize the cells lining the intestinal tract of insects, only a few actually being pathogenic for their insect host, among these particularly the typhus agent, which is pathogenic for the louse. They may be transmitted to an animal host either in the feces or the saliva of this vector. In the United States the most important rickettsial infections of man are typhus fever and Rocky Mountain spotted fever, the first being transmitted by lice, the latter by wood ticks. Cases of true or epidemic typhus are rare in this country, but the related disease, endemic typhus or murine typhus, does occur, particularly in the Southern states. It is caused by *R. mooseri* and is spread by a rat flea rather than body or head lice. The rickettsiae of Rocky Mountain spotted fever can be transmitted from the female tick through the eggs to the progeny. Tsutsugamushi, primarily an Oriental disease common in swampy areas and rice fields, is caused by *R. nipponica* (*R. tsutsugamushi*), is spread by infected mites, and in its clinical symptoms is closely related to typhus and Rocky Mountain spotted fevers. The fourth common rickettsial infection, Q fever, caused by *R. burnetii* (or *Coxiella burnetii*), is clinically distinct from the three above-mentioned diseases. Q fever rather closely resembles influenza or atypical pneumonia in its clinical picture. The characters of the rickettsiae and rickettsial infections are best illustrated by typhus fever.

Typhus fever has been known for many centuries, and as Zinsser has pointed out in his book "Rats, Lice and History," it has markedly influenced the course of history. It becomes epidemic when peoples are crowded for lengths of time under unhygienic conditions in close contact, e.g. in armies, when cleanliness becomes impossible to maintain, and in poverty-ridden, starving populations. Insecticides such as DDT greatly reduce the danger of outbreaks of typhus if carefully employed to control the louse population of man. Their worth was clearly demonstrated in occupied countries during the Second World War. Some control of the

spread of this disease and the closely related rickettsial diseases is possible by the use of vaccines prepared from chemically killed rickettsiae produced in cultures in the developing chick embryo in hen's eggs. Control of other rickettsial diseases can be accomplished to some extent by control of the insect vector and with vaccines.

The Bartonellaceae are primarily parasites of erythrocytes in man and other vertebrates but may be transmitted by arthropod vectors in some cases. They cause bartonellosis in man and related infections in lower animals. These organisms appear to be more closely related to the bacteria than are the Rickettsiaceae, since the Bartonellaceae are known to multiply by binary fission and some species have been cultivated in serial passage in cell-free media.

The third family of the Rickettsiales, the Chlamydozaceae, consists of organisms which are small, pleomorphic, often coccoid in shape, obligate intracytoplasmic parasites with characteristic development cycles. Many of these organisms were once, or still are, believed to be viruses. They are the cause of infectious diseases such as trachoma, conjunctivitis, psittacosis (parrot fever), one type of viral pneumonia, a venereal infection known as lymphogranuloma venereum, and a number of infections of lower animals.

Classification of these borderline organisms is difficult, and an extensive study of them would be out of place in general bacteriology. They have been considered primarily to introduce the viruses and to indicate that bacteriology or microbiology is not a stagnant field but rather one in which many of the members are not well known. It might be of interest to mention that similar organisms, possibly rickettsiae or bacteria, which are parasitic upon other microorganisms, protozoa, are known and in Bergey's Manual (sixth edition) were tentatively placed in an appendix to the order Rickettsiales. With increasing studies of the Rickettsiales and of the Borrelomycetaceae our knowledge of these organisms in the future will not be as incomplete as at the present time, and they can be considered more systematically.

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## CHAPTER 7

### THE VIRUSES

There is no single criterion by means of which the filtrable viruses, or more simply the viruses, can be differentiated from the bacteria and the forms of life intermediate between the bacteria and the viruses. The viruses and intermediate organisms are characterized primarily by their parasitic mode of life, their minute size, and their inability to multiply well, if at all, on lifeless media. An attempt was made to define and classify these various agents in the sixth edition of "Bergey's Manual of Determinative Bacteriology" in a supplement described as "groups whose relationships are uncertain." This classification can be summarized as follows:

1. Obligate intracellular parasites or dependent directly on living cells

- a.* Not ultramicroscopic and only rarely filtrable. More than  $0.1\ \mu$  in diameter

*Group I. Order Rickettsiales*

- aa.* Usually ultramicroscopic and filtrable. Except for certain pox viruses of animals and a few plant viruses, less than  $0.1\ \mu$  in diameter

*Group II. Order Virales*

2. Grow in cell-free culture media with the development of polymorphic structures including rings, globules, filaments, and minute reproductive bodies (less than  $0.3\ \mu$  in diameter)

*Group III. Family Borrelomycetaceae*

We saw in the preceding chapter that the pleuropneumonia group and the rickettsiae, the organisms intermediate between the bacteria and the viruses, together with the viruses are included in the Schizomycetes in the seventh edition of Bergey's Manual. The viruses are classified as one order, the Rickettsiales as another, in the class Microtobiotes or smallest life, but the viruses are not considered individually in the first volume of the seventh edition. No system of classification has been accepted generally, and viruses are most frequently designated by common names. It has been proposed that viruses be given binomial names, the first indicating the group to which the virus belongs and the second name that of the individual virus, the terms "group" and "group member" being employed rather than genus and species. The virus of smallpox would be

termed Poxvirus variolae, that of poliomyelitis (infantile paralysis) Poliovirus hominis, and that of influenza A virus, Myxovirus influenzae-A, according to this system. Undoubtedly other proposals regarding nomenclature and classification will be made before a satisfactory system is developed.

The viruses are not readily set apart from the Rickettsiales and the Mycoplasmatales by definition, and some workers classify certain infectious agents, e.g., those of psittacosis and lymphogranuloma venereum, as viruses while others consider them as Rickettsiales. For our purposes we can define viruses as *ultramicroscopic, filtrable, infectious agents which depend on living plant or animal cells for their multiplication*. Rickettsiae also depend upon other cells for their reproduction, but with one exception they are not filtrable agents. The pleuropneumonia organisms tend to pass through filters more readily than certain of the larger viruses, but the latter have not been cultivated on lifeless media. Both viruses and the rickettsiae tend to lead an intracellular existence in the hosts upon which they are parasitic, but this is also true of certain bacteria, particularly of the genus *Brucella*. The bacteria, however, can be cultivated in the laboratory on lifeless media. There is considerable heterogeneity amongst the agents recognized as viruses, and no definition as yet advanced is completely satisfactory.

Pasteur in his studies on rabies was unable to demonstrate the presence of a visible causative agent in brains or spinal cords from rabid animals, such tissue, however, being infective for susceptible animals. Pasteur suggested that infectious agents might possibly exist below the limits of visibility in the microscope. In 1892, Iwanowski, working with a mosaic disease of tobacco plants, demonstrated that juice from infected plants remained infective after passage through bacteria-retaining filters. Little attention was paid to this observation until 1898 when Beijerinck made a similar observation. Because he was unable to demonstrate, culturally or microscopically, the presence of bacteria in the infectious juice and because the agent diffused through agar, Beijerinck concluded that the fluid itself, which he spoke of as a "living fluid contagium," must be infectious. In the same year Loeffler and Frosch reported the filtrability of the agent of foot-and-mouth disease of cattle and Sanarelli suggested that a tumor-like malady of rabbits was of virus origin. Later numerous other diseases were recognized as being of virus origin, and during the First World War Twort and particularly d'Hérelle recognized that bacteria also are subject to infection with filtrable viruses called bacteriophages.

These various discoveries brought forth many ideas concerning the nature of the filter-passing, ultramicroscopic agents, since they opened an entirely new field of biology on the border line between animate and



inanimate matter. Are the viruses biological or chemical entities? Unlike known chemical poisons or toxins, they elicit multiplication of themselves when present in susceptible cells; unlike the bacteria, they are not visible (with a few exceptions) under ordinary microscopes and do not multiply on lifeless media. In 1935 Stanley was able to crystallize a protein from the juice of plants infected with tobacco-mosaic virus; this



Fig. 7-1. M. W. Beijerinck.

crystalline material was highly infectious, and its infectivity remained constant on repeated crystallizations. Since then, other viruses have been crystallized or obtained in highly purified form, and each is composed of nucleoprotein alone or in association with other substances.

Tobacco-mosaic virus is one of the smallest viruses (see Figs. 6-8 and 7-2), being approximately 15 millimicrons ( $m\mu$ ) in diameter by 280  $m\mu$  in length. Vaccinia virus is one of the largest viruses, approximately 225  $m\mu$  in diameter, and in addition to nucleoprotein has been found to contain lipid, carbohydrate, and the enzymes or coenzymes phosphatase, catalase, lipase, biotin, ribo-

flavin, flavin adenine dinucleotide, and an as yet unexplained but apparently significant amount of copper. Vaccinia and many other viruses studied have an appreciable water content, while tobacco mosaic virus crystals are free of water, suggesting in the latter complete lack of metabolic activity. Yet these particles, when introduced into the susceptible host, can alter the activity of the cells in which they become established and lead to multiplication of the virus to hundreds or even thousands of times the amount present in the original inoculum.

As a whole, on the basis of information now available, the viruses increase in chemical complexity and in internal structure (as evidenced by electron micrographs) as they increase in size from the smaller ones—viruses of poliomyelitis and tobacco mosaic—through intermediate sizes—represented by influenza and bacterial viruses—to vaccinia and variola (see Fig. 7-2 and Table 7-1 on pages 148-149). Chemical complexity and size of a virus such as that of tomato bushy stunt are little or no greater than those of chemical entities such as hemoglobin or certain hemocyanins, while vaccinia virus approaches bacteria and related micro-

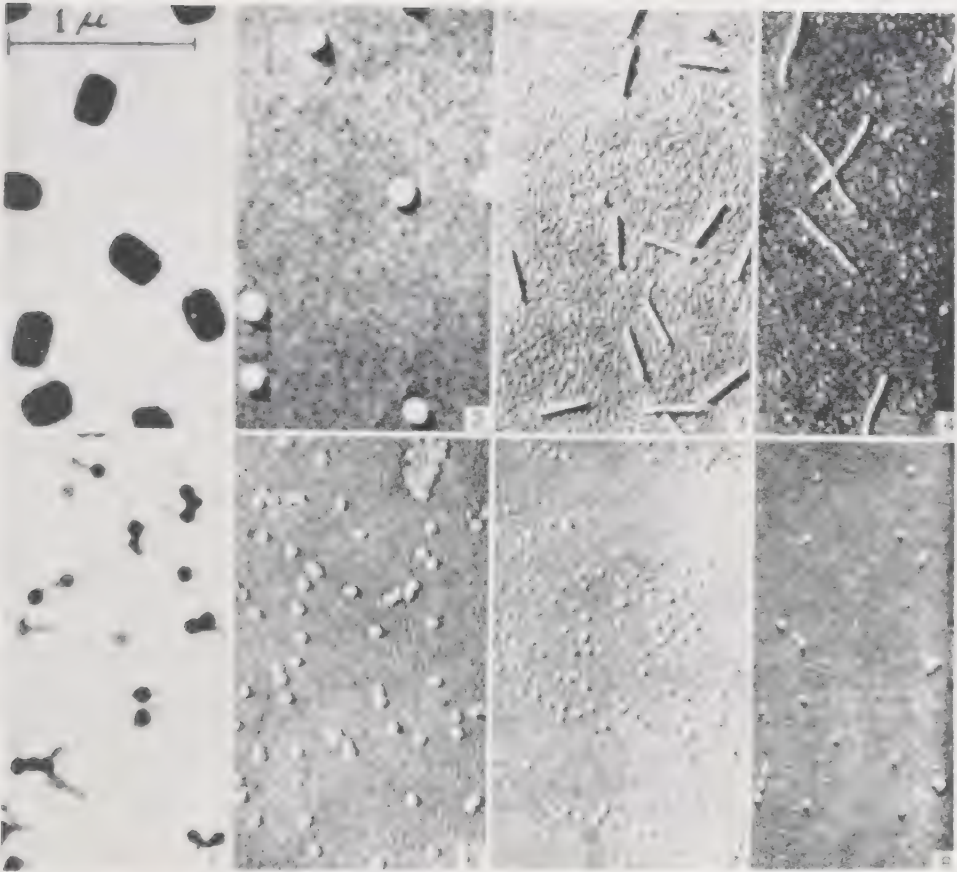


FIG. 7-2. Electron micrographs of different viruses enlarged to the same extent. (1) Vaccinia, (2) Influenza, (3) Tobacco mosaic, (4) Potato-X, (5) T2 Bacteriophage, (6) Shope papilloma, (7) Southern bean mosaic, (8) Tomato bushy stunt. [From Knight, *Nucleoproteins and virus activity*, Cold Spring Harbor Symposia on Quantitative Biology, **12**, 115 (1947).]

organisms in these respects. If it could be cultivated in lifeless media, no doubt it would be recognized as a microorganism. Much remains to be learned about these infinitely minute infectious agents on the lower border line of biology, agents which may be chemical entities or complexes which borrow characteristics of life from the cells which they parasitize and in the absence of their host exhibit properties of inanimate matter. Whatever their real nature we can learn much from a consideration of their general properties.

Green and Landlaw have suggested that the viruses represent degenerate descendants of pathogenic microorganisms. One of the more convincing arguments is that various degrees of degeneration are represented in the viruses, ranging from a fairly complete biological entity such as

TABLE 7-1. CHARACTERISTICS \* OF SOME VIRUSES PATHOGENIC FOR MAN

Disease	Size (m $\mu$ ) and shape	Portal of entry	Tissue preferred
<i>Dermatropic</i>			
Vaccinia (cowpox) . . . . .	235 $\pm$ , spherical or brick-shaped	Skin	Skin
Variola (smallpox) . . . . .	200 $\pm$ , spherical or brick-shaped	Respiratory tract or skin	Skin and mucous membranes
Chickenpox (varicella) . . . . .	240 $\pm$ , brick-shaped	Respiratory tract	Skin and mucous membranes
Measles (rubeola) . . . . .	100?, spherical	Respiratory tract	Respiratory epithelium and skin
German measles (rubella)	?	Respiratory tract	Skin and cervical lymph glands
Herpes simplex . . . . .	150 $\pm$ , spherical	Usually present in tissues, activated by fever, etc.	Skin, mucous membranes, and nerves
<i>Neurotropic</i>			
Poliomyelitis . . . . .	30 $\pm$ , spherical?	Pharyngeal mucosa and intestinal tract	Central nervous system
Rabies . . . . .	125 $\pm$ , ?	Nerve fibers or blood stream	Central nervous system
St. Louis encephalitis . . . . .	20-30	Blood stream(?)	Central nervous system
Equine encephalomyelitis	30-50, spherical	Blood stream, nose(?)	Central nervous system
Herpes zoster (shingles)	240 $\pm$ , brick-shaped	Skin and mucous membranes	Skin and nerves
<i>Viscerotropic</i>			
Yellow fever . . . . .	22 $\pm$	Blood stream	Liver
Infectious hepatitis (epidemic jaundice) . . . . .	?	Blood stream	Liver
Colorado tick fever	25 $\pm$	Blood stream	Spleen(?)

\* Based in part on a summary in a supplement to *Science*, 3, no. 11 (1953) published by the Upjohn Company.

TABLE 7-1 CHARACTERISTICS OF SOME VIRUSES PATHOGENIC FOR MAN (Continued)

Disease	Size (m $\mu$ ) and shape	Portal of entry	Tissue preferred
<i>Pantropic</i>			
Coxsackie group.....	20 $\pm$	Nose, pharynx, or intestine(?)	
Dengue fever.....	20 $\pm$ , dumbbell- to rod-shaped	Blood	
<i>Miscellaneous</i>			
Influenza.....	100 $\pm$ , ovoid to spherical	Respiratory tract	Respiratory epithelium
Common cold.....	Less than 50(?)	Nose	Nasal mucosa
Epidemic keratoconjunctivitis.....	75 $\pm$	Conjunctiva	Conjunctiva
Newcastle disease virus conjunctivitis	115 $\pm$ , filamentous or spermlike	Conjunctiva	Conjunctiva
Mumps.....	180 $\pm$ , round to oval	Mouth	Salivary glands, gonads, central nervous system
Lymphogranuloma venereum (a virus?)	300 $\pm$	Genital tract, skin(?)	Lymph glands

vaccinia to the smallest and apparently simplest viruses. Possibly in the latter case all that is left is an aberrant gene, but the possibility remains that the virus may be an aberrant gene of the host rather than of the parasite species. The above discussion indicates that speculation is rife in the virus field, and the ideas presented should be considered as speculative in character. To return to more solid ground, it might be well at this time to summarize briefly the main facts known concerning the viruses and then to illustrate the nature of viruses and of their action by a consideration of the bacterial viruses, or bacteriophages.

**Properties of Viruses.** There are certain properties common to all the agents regarded as viruses, and these may be summarized as follows:

1. Viruses are obligate parasites, multiplying only in the presence of susceptible cells.
2. They are specific in that a virus naturally attacks but one or a limited number of host species and generally specific tissues or cells within the host, i.e., they exhibit definite tropisms.



3. When a virus is introduced into a susceptible host, multiplication of the virus occurs with production of more of the same agent but subject to variation (see Chap. 14) within limits similar to those observed with higher organisms.
4. They are susceptible to general cellular poisons, in many instances being somewhat more resistant than the bacteria to disinfectants. Many of the animal viruses, however, are more susceptible to inactivation by oxygen or oxidation than are the common bacteria. Most viruses, except the large borderline ones, are resistant to the antibiotics.
5. They are particulate as demonstrated by ultrafiltration, ultracentrifugation, diffusion, and electron microscopy, ranging in diameter from approximately 10 to 300 m $\mu$ . The majority of the viruses appear to be more or less spherical in shape.
6. They are antigenic, eliciting the production of antibodies capable of neutralizing their activity. All viruses that have been purified and chemically analyzed are composed of protein and nucleic acid or these substances plus others in the larger viruses. As a general rule the nucleic acid is of the ribonucleic acid type in the plant viruses, deoxyribonucleic acid in animal ones. However, a number of exceptions to this statement are known, e.g., tobacco mosaic virus contains deoxyribonucleic acid and the viruses of influenza and poliomyelitis, ribonucleic acid.
7. Viruses are recognized by what they do and not by what they are. Their destructiveness in both the plant and animal kingdoms is comparable in importance with that of the bacterial pathogens, and the recognized number of species (248) in Bergey's Manual (sixth edition) exceeds those of any order of the Schizomycetes with the exception of the Eubacteriales (929).

**The Bacteriophage.** Certain specific viruses pathogenic for plants or animals will be considered briefly in this and later chapters, but at this time attention will be focused on the group of viruses active against the bacteria, as this group well illustrates many of the agents studied in virology. In addition, the student of general bacteriology can readily study the phenomena of bacteriophagy in the laboratory, since they can be demonstrated in a short period of time with simple equipment and without resort to the use of experimental plants or animals.

A bacteriophage or bacterial virus is a species of virus parasitic upon specific bacteria, generally a particular species thereof. Frequently the specificity is even greater, the phage attacking one or more strains within a species. A considerable number of phages have been isolated, generally from fecal material, and they have been differentiated from each other on the basis of strain or species specificity for the bacteria attacked by the phage. Just how the bacteriophage acts upon the bacteria is not definitely known, but the end result is complete dissolution, lysis, of the susceptible bacteria, hence the name bacteriophage, which literally means bacteria eater. It was hoped that the phages would be of value as therapeutic agents, but clinical tests generally indicated that phage was not active in vivo.

D'Hérulle believed that bacteriophages are autonomous, ultramicroscopic parasites which possess the power to invade susceptible cells, multiply therein, and in time bring about the lysis of the invaded cell.

Other workers have considered them to be chemical agents, possibly enzyme in character. As is true for all the viruses, there is no completely satisfactory explanation of their nature, an inherent difficulty being the lack of a comprehensive definition of *life* and therefore of satisfactory criteria for distinguishing between animate and inanimate matter. Certain bacteriophages have a diameter (8 to 10  $m\mu$ ) which is within the upper limits of molecular size; others with a diameter of 100 to 150  $m\mu$  approach the lower limits of size of agents assumed to be living on the basis of our common criteria of life. (The size of various bacteriophages has been determined by ultrafiltration and other physicochemical techniques and from electron micrographs.)

The electron microscope has proved to be a valuable tool in the study of the morphology of bacteriophages. Some have been found to be more or less spherical bodies with no indication of internal structure; others are somewhat elongated, and a few have been shown to possess definite head- and tail-like structures, with possible differentiation within the head (see Figs. 7-3 and 7-4). In other words, as is also true for the viruses as a group, the structure of some bacteriophages suggests an organismal rather than a molecular type of configuration.

If a small amount of feces-containing material or of ground-up insects is dispersed in broth and passed through a bacteria-retaining filter, the filtrate will frequently be found to contain one or more bacteriophages.

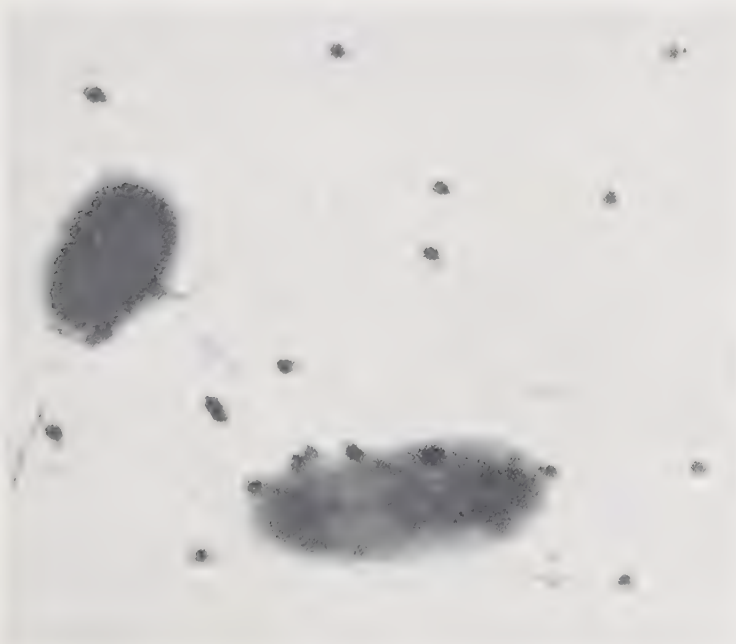


FIG. 7-3. Electron micrograph of *Pseudomonas aeruginosa* and of a bacteriophage against it. (Courtesy of E. W. Schultz.)



FIG. 7-4 Electron micrograph of the T2 anticoliform bacteriophage ( $46,000\times$ ) showing the tadpole-like structure. (Courtesy of C. E. Schwerdt and the Virus Laboratory, University of California.)

Assume that we are interested in isolating a bacteriophage active against *Escherichia coli*. The next procedure would be to add a drop or two of the filtrate to a freshly inoculated broth culture of *E. coli* and to incubate this mixture and one control tube of broth inoculated with the same number of bacteria and at the same time as the phage test culture. If an anti-*coli* phage were present in the material tested, the control tube would be cloudy after six to ten or more hours' incubation, while the test culture would be clear. The same phenomenon should be evident when a drop or two of the phage test culture is added to another freshly inoculated broth culture of *E. coli*, and so on in series indefinitely. At times clearing may not be entirely complete, as a few individual bacterial cells in the culture might be resistant to the action of the phage, but the presence of phage can be demonstrated in cell-free filtrates of the phage-containing culture. In time the resistant organisms give rise to what is



known as a "secondary culture." Many of the organisms in such secondary cultures remain phage-resistant, a condition analogous to but not identical with the resistance to infection developed in strains of certain species of plants. (A number of technical or biological difficulties are frequently encountered in work with these bacterial viruses, time and temperature of incubation, nature of the medium, size of inocula of phage and bacteria, and nature of the phage and bacterial species influencing the results of a given test. This discussion of bacteriophagy is simplified to indicate the results obtained under favorable conditions.)

The lytic action of bacteriophage can also be demonstrated on a solid medium, the surface of which has been inoculated with a suspension of the susceptible bacterium and with a small number of bacteriophage particles active against the particular bacterium. A control plate, inoculated with the same amount of the bacterial suspension alone, will show profuse and more or less solid growth after incubation, while here and there in the growth on the phage-containing plate there will be clear, glassy, pinhole areas known as *plaques* in which no bacterial growth is evident (see Figs. 7-5 and 7-6). These plaques can be regarded as colonies of bacteriophage which have developed at the expense of the bacteria originally present in their neighborhood. Each plaque may contain many millions of bacteriophage particles. Knowing the dilution of the original phage-containing suspension and the amount of this dilution added to the agar, and from a count of the number of plaques which developed on the agar, it is a simple arithmetic process to calculate the original concentration of the phage. It is a method analogous to the dilution and colony-count procedure for the determination of the numbers of bacteria in a sus-

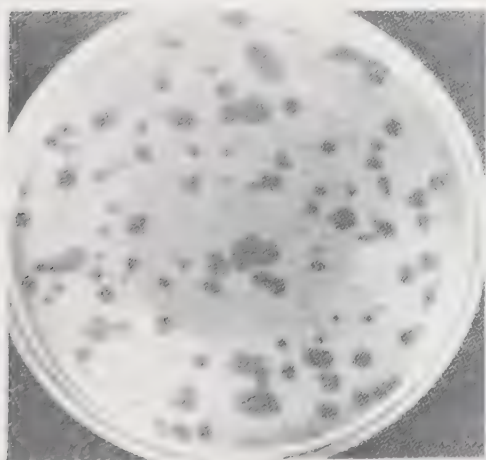


FIG. 7-5. Bacteriophage plaques, clear areas in an otherwise continuous growth of *Escherichia coli*.

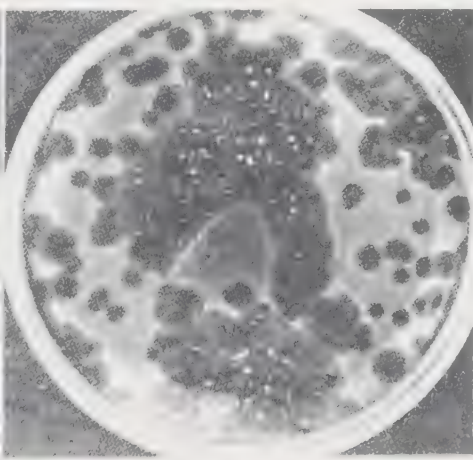


FIG. 7-6. Secondary growth of *Escherichia coli* in plaques of a bacteriophage active against the original strain.



pension (see Chap. 12). The calculation can be illustrated as follows: Suppose that 1.0 ml. of a  $1:1,000,000$  ( $10^{-6}$ ) dilution of the bacteriophage suspension was plated on the surface of the inoculated agar and that 207 plaques developed. One milliliter of the  $10^{-6}$  dilution, therefore, contained 207 phage corpuscles, and since there would be 1,000,000 times as many in the original suspension, the original number was 207,000,000. When somewhat greater numbers of phage particles are present, the plaques will run together, and it will be impossible to obtain an accurate

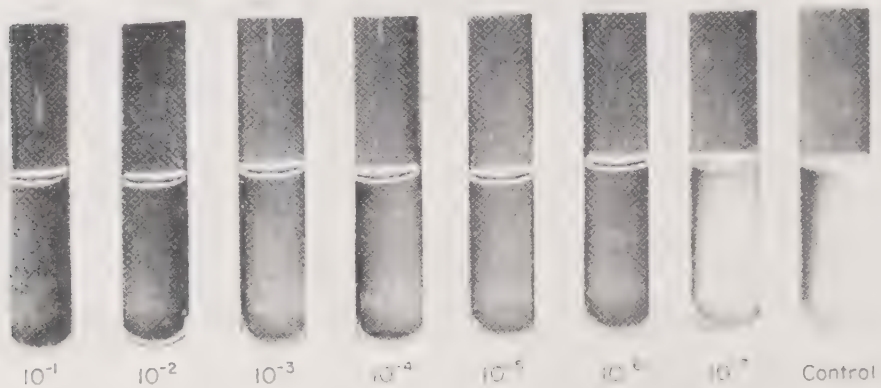


FIG. 7-7. Titration of a bacteriophage suspension. Lysis is observed (broth is clear) in dilutions through  $10^{-6}$  but not in  $10^{-7}$ , thus indicating a concentration of around 1,000,000 phage particles per milliliter of the phage suspension added to the first tube. The tube on the right serves as a control to indicate extent of growth in the absence of phage.

count. When still greater amounts of phage are added to the plates, no individual plaques will be evident, just as no separate colonies of bacteria are observed when too large inocula are employed.

At times isolated bacterial colonies show irregularities in their margins, the colonies appearing as though they had been "nibbled" by some agent. These nibbled or moth-eaten colonies are generally due to the lysis of phage-susceptible bacteria developing in a phage-containing colony of a bacterial strain at least partially resistant to the lytic action of the particular bacteriophage. Genetic factors appear to control the development of phage-resistant or phage-susceptible strains of bacteria. Phage-resistant colonies of bacteria frequently develop within plaques or larger areas where lysis had been evident (see Fig. 7-6), and the cells tend to remain resistant. Species of bacteriophage may likewise undergo variation or change, which is transmissible in a manner apparently analogous to the transmission of hereditary characters in material known to be living (see section on bacterial variation, Chap. 14).

In addition to the lytic type of bacterium-phage relationship, many strains of bacteria carry phage from generation to generation without noticeable lysis. Generally a few of the cells undergo lysis, the presence of the phage so released being recognizable by ordinary lytic tests if another strain of bacteria susceptible to the phage is available. Phage-bearing cultures of this nature are known as *lysogenic cultures*, while phages that have established a lysogenic relationship with a bacterial host are called *temperate phages*, in contrast to the *virulent phages* which induce lysis. The lysogenic relationship is an example of skillful parasitism, the virus being maintained indefinitely with little or no damage to the host. A somewhat similar relationship may be noted in man, the virus of herpes simplex being present in the body possibly throughout life. However, it becomes evident only at times, e.g., following trauma or fever, when the virus is activated and produces damage indicated by the development of fever blisters.

If one inoculates a suitable medium with a definite number of phage particles and of bacteria, it is possible to follow the increase or decrease with time in numbers of phage particles and of bacteria. It will be observed that the numbers of bacteria increase with time more or less parallel to the increase observed in a phage-free culture. After some time, depending upon the initial numbers of phage and bacteria, the numbers of bacteria in the phage-containing culture will no longer increase parallel to the control culture and shortly thereafter will rapidly decrease and approach zero as lysis occurs. The increase in concentration of phage is generally more rapid than that of bacteria, since each bacterium infected with a phage may give rise to more than one hundred phage corpuscles on lysis. Only one phage particle need enter a bacterium to be effective in establishing an infection. Phage particles liberated on lysis of the cell may infect phage-free bacteria, and the process will continue until all have been infected and lysed.

Quantitative studies on the relationship between bacteria and their viruses, studies on the chemical composition of the bacterial viruses, determination of alterations in the metabolism of bacteria induced by phage, and electron microscope observations have led to a fairly clear picture of bacteriophagy. Most studies have been carried out with a series of seven phages ( $T_1$ ,  $T_2$  . . .  $T_7$ ) active against a common host, *Escherichia coli*, strain *B*. Some differences are noted, but the general behavior can be summarized as follows: These phages are sperm-like in appearance and when added to a young culture of their host, a phage particle (particles) is (are) adsorbed by a bacterial cell, the tail-like portion of the phage uniting with the bacterium. The phage particle is composed of a core of deoxyribonucleic acid surrounded by a protein coat. Following adsorption of the phage, the deoxyribonucleic acid penetrates

into the cell, the protein coat remaining behind and playing no further role in the events that follow adsorption and the subsequent taking up of the nucleic acid by the cell. There is some evidence that the phage deoxyribonucleic acid may fragment in the cell and more evidence that it does come into contact with nuclear material of the host. Marked changes in the nuclear apparatus of *E. coli* can be noted shortly after infection of the cell has occurred. Bacterial growth is suppressed and the synthetic machinery (enzymes) of the cell is shunted from the production of bacterial substance to the synthesis of phage protein and nucleic acid. The phage deoxyribonucleic acid in some manner becomes the directive force for the activities of the cell. Phage nucleic acid and protein are synthesized separately and only combine to form complete particles of phage late in the course of events. The particles do not multiply as such, in contrast to the multiplication of bacteria themselves. With the aid of tracer elements it has been established that the many "descendants" of the original phage particle contain little material derived from the infecting particle and, for the most part, are formed from material in the cell at the time of infection and also from constituents of the medium. Little is known of the release phase of bacteriophagy—that period of time during which the majority of the phage particles are organized in their final form and released by lysis of the cell. It is apparent that a phage, or rather phage material, so alters the metabolism of a sensitive host that activities of the cell are shunted from the formation of bacterial substance to the synthesis of bacterial virus components. Phages induce, and are products of, altered metabolism of the phage-infected cell. This might be true for the viruses in general and, if so, indicates a marked difference between bacterial and viral infections. Bacteria use tissue or cellular constituents as food, converting them into bacterial matter, while viral nucleic acid alters the metabolism of the host cells in such a manner that the infected cells produce viral substance.

**Tobacco Mosaic Virus.** We have seen that this virus was the first one to be detected and the first one to be crystallized. This virus enters the tobacco or other susceptible plants through broken hairs or wounds on the leaves. During the development of the infection, the leaves curl and exhibit an irregular mosaic pattern of different shades of green. The virus does not kill the plant but it inhibits growth, reducing the yield and quality of the crop. Relatively little is known concerning the actual mode of increase in concentration of the virus. The virus can be demonstrated in all parts of the infected plant. From the diseased plant material can be separated that contains only protein and ribonucleic acid characteristic of the virus. Physical and chemical studies indicate that this virus material exists as rod-shaped particles made up of a more or less hollow core surrounded by a protein coat in which the ribonucleic



acid appears to be embedded in helical strands along the long axis of the particle. It has been reported that the nucleic acid is infectious by itself. In this respect it resembles the infection established by phage deoxyribonucleic acid injected into the bacterium from the phage particle. This raises the question of why a ribonucleic acid appears to be the directing agent of some viruses and deoxyribonucleic acid of others. No entirely satisfactory answer has been advanced. A considerable number of viruses infectious for plants are known.

**Human Viruses.** We have seen that the nutritional requirements of viruses are such that they can be supplied only by specific living cells and that these cells also provide most or all of the enzymes required for the synthesis of viral material. Viruses do not respire as do bacteria, and in general are inert particles except when present within susceptible cells. In the early years of bacteriology the use of susceptible animals was mandatory for the study of the human and other animal viruses. Today many of the viruses have been cultivated in tissue cultures and in the developing hen's egg, thus making study of these viruses somewhat easier. Viruses are cultivated to a considerable extent today in fertile eggs, the virus inducing the death of the embryo in the case of encephalitis, the production of plaques or pocks on the chorioallantoic membrane (see Fig. 7-8), the development of hemagglutinins (influenza), and the production of infective virus in most instances where the fertile egg is a satisfactory medium. Tissue cultures of monkey kidney tissue are em-

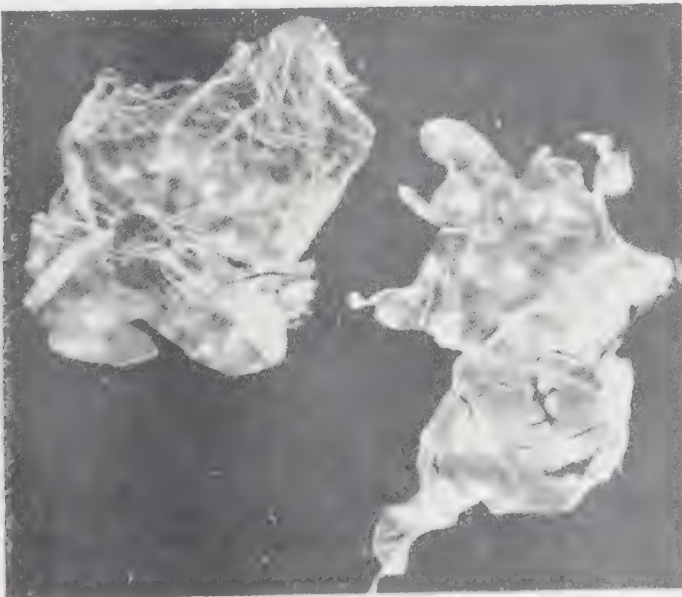


Fig. 7-8. Plaques or pocks (light areas) induced by the growth of vaccinia virus on the chorioallantoic membrane of the developing chick embryo. (Preparation courtesy of D. F. Roman.)



ployed for the production of poliomyelitis virus on the large scale necessitated by the development of an apparently successful vaccine (see Figs. 7-9 and 7-10).

The virus of poliomyelitis has been obtained in a highly purified form and consists of spherical particles of protein plus ribonucleic acid. Three antigenic types (I, II, and III) have been recognized, and successful



FIG. 7-9. Poliomyelitis virus cultivated in tissue culture. The fluid is removed from these culture bottles and becomes the virus pool to be inactivated for use in the vaccine. (Courtesy of Eli Lilly and Co.)

FIG. 7-10. Roller tube cultures shown here are used for determinations of the concentration of poliomyelitis virus in cultures such as shown in Fig. 7-9. All cultures are maintained in incubator rooms in which temperature and humidity are carefully controlled. (Courtesy of Eli Lilly and Co.)

immunization requires the use of all three types in a vaccine. Early ultrafiltration and ultracentrifugation studies indicated that the particles were 8-12  $m\mu$  in diameter, but electron micrographs (see Fig. 7-11) show that the MEF1-strain virus particles are 27-31  $m\mu$  in diameter.

Most work has been carried out with the virus of influenza of which three major antigenic types, A, B, and C, are known. The A type in particular is quite variable. Burnet (1956) concludes that the influenza virus particle is not so rigidly organized as are the bacterial viruses and the smaller viruses such as those of tobacco mosaic and poliomyelitis. The virus particles are spherical and have a diameter of approximately 100  $m\mu$ , but the virus apparently can also exist in the form of filaments. Purified virus is composed of 20-30 per cent lipid, 5 per cent carba-

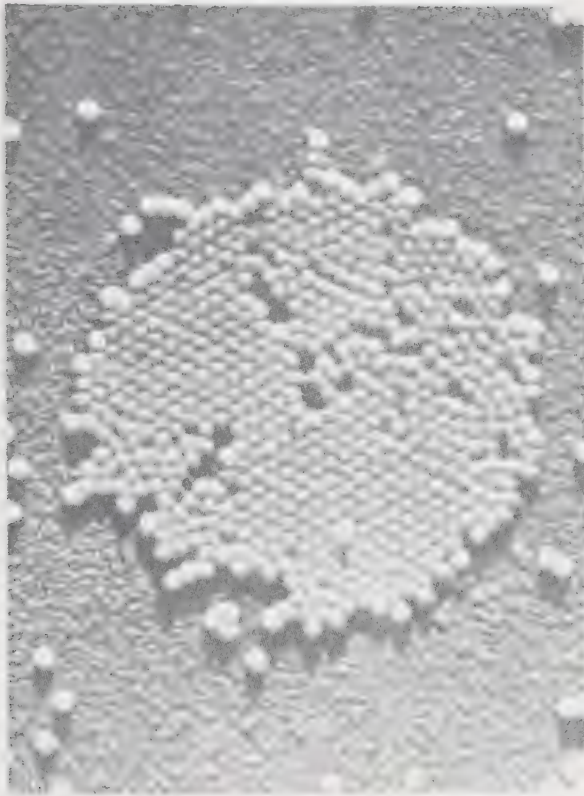


FIG. 7-11 Electron micrograph of a purified suspension of poliomyelitis virus. The particles are 27 mμ in diameter and are shown here magnified 75,000 times. (Courtesy of C. E. Scheraga and the Society for Experimental Biology and Medicine.)

hydrate, 60-70 per cent protein, and around 1 per cent ribonucleic acid. There is some evidence that the particle contains host cell material as well as material characteristic of the virus itself. Burnet believes that the virus is a loosely organized unit in which a surface membrane, derived largely from the host cell but containing viral material as well, encloses a variable number of genetic determinants of the ribonucleic acid type together with some material from the host cytoplasm.

It has been established that when influenza virus is inoculated into the allantoic cavity of the chick embryo, the infective particles attach themselves to the free surface of susceptible cells. This union is mediated by attachment of an enzyme-like component of the virus to prosthetic groups of mucoprotein on the cell surface. (Similar mucoproteins are present on the surface of red blood cells, and some viruses in suspension can be recognized by the fact that the virus unites with these cells and causes them to clump together—the phenomenon of hemagglutination). After adsorption the virus particle enters the susceptible cell and loses its identity as an infective particle. About three hours after infection,

virus can be detected in the cell, and in about another hour infective virus is liberated from the cell. The virus in some manner appears to take over partially or completely the protein- and ribonucleic acid-synthetic systems in the cell which lead to the synthesis of viral material. Replication of the virus particles may take place at the cell surface. In many respects the formation of influenza virus resembles the formation of bacterial viruses in bacteria. Whether this is a general type of mechanism by which other viruses are formed in susceptible cells remains to be determined. Some of the more common viruses and viral diseases will be considered in the chapters on infections of man, animals, and plants.

**Recapitulation.** In the last two chapters we have considered in a general and somewhat simplified manner the main characteristics of various, primarily parasitic, agents on the border line between animate and inanimate matter. The pleuropneumonia group was characterized as a variety of organisms approaching the lower limits of visibility in the microscope, the majority of the species being parasitic in character and differing from the bacteria in their manner of multiplication, their poor staining qualities, their need for relatively high concentrations of serum for growth, and their lack of a rigid cell wall with consequent ability to "flow through" the pores of a bacteria-retaining filter.

The rickettsiae in many respects closely resemble the smaller bacteria with the exception of their staining characteristics and, above all, their entire dependence upon certain, quite specific, living cells as a suitable pabulum for multiplication. The pleuropneumonia group and the rickettsiae may be looked upon as transition groups between the bacteria proper and the viruses, parasitic agents of submicroscope size. Most of the agents in these three groups are entirely dependent upon living cells for multiplication, although they can remain active for long periods of time away from their host if stored under proper conditions. It is known that viruses possess the ability to increase in amount and to undergo variation under appropriate conditions and that they are particulate. But it has not been definitely established that they respire or that they respond to external stimuli in a manner analogous to living cells. In other words they exhibit some, but not all, of the characteristics of living matter as exhibited by cells such as the bacteria and protozoa. Some of the larger viruses appear to approach the pleuropneumonia group or the rickettsiae in their complexity, others to resemble complex proteins more closely. There is no doubt as to their existence and to the fact that they present many problems of interest and of challenge to the microbiologist. Perhaps it might be well to conclude, as Rivers has done, that three possibilities exist concerning the viruses: (1) the smaller viruses may be special inanimate pathogenic agents; (2) the medium-sized viruses may

represent primitive or at least unknown forms of life; and (3) the larger viruses may be the midgets of the known microbial world.

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## CHAPTER 8

# ENERGETICS, METABOLISM, AND NUTRITION

Bacteria, like other living cells, may be considered to have three general attributes of life:

1. The conservation of a distinctive, particulate form
2. The ability to use energy
3. The power of adaption

We have considered that the individual species of bacteria possess and maintain a distinctive, particulate form characteristic of the species, but this does not mean that the cells of a given species cannot change in size or shape. Indeed, variation in morphology is frequently observed, particularly with age of the culture or with change in the nature of the environment. The cells tend to maintain a distinct, particulate form under a given set of conditions, but as conditions change, the cells adapt themselves to that change either morphologically and or by their ability to use different sources of energy and of building material. This power of adaption is limited by inherent characters of the cells.

At this time we want to consider the second characteristic of living matter, the ability to use energy. The development of the concept of energy and of its utilization by the cell requires some abstract reasoning, and the discussion to follow is presented to arouse thought concerning the energetics of microbial growth and activities and not as material to be memorized by the student. It has been well said that the struggle for existence is a struggle for energy. The degradation of matter by microorganisms, or by cells in general, is an indication of their constant need for energy to maintain life. Fermentations, so characteristic of bacterial life, and oxidations in general are simply chemical mechanisms for releasing energy from matter and making some of it available to the cell. Many of the energy-providing reactions are carried out in the cellular protoplasm, protoplasm being regarded as a complex colloidal system, composed of various organic and inorganic substances, which provides suitable conditions and mechanisms for the complicated chemical reactions essential for maintenance of the cell. All the chemical reactions involved in the life of the cell whereby food is transformed into cellular

material or utilized as a source of energy for the performance of chemical, mechanical, electrical, and other work are grouped under the general term *metabolism*. These reactions are spoken of collectively as the metabolic activities of the cell. Metabolism thus may be defined as *the sum of all chemical changes, both assimilatory and dissimilatory, induced by an organism*. The term *nutrition* is also frequently employed more or less synonymously with metabolism, although with microorganisms the term nutrition refers primarily to the food requirements of the organism. The term food, as it will be employed here in its widest sense, implies all substances which, when taken into an organism, serve as sources of energy or of building material.

The metabolic activities of living matter are controlled to a great extent by enzymes. *Enzymes* can be defined as *proteinaceous agents produced by the cell which act as catalysts of specific reactions*. Each species of bacteria possesses somewhat different batteries of enzymes or different relative amounts of particular enzymes, and indirectly the finer details of bacterial classification on the basis of biochemical activities, such as fermentations of sugars, reduction of nitrate, production of hydrogen sulfide, etc., are based on the enzymic constitution of the species. This is illustrated in Table 8-1, in which typical biochemical activities of a number of bacterial species are listed.

The metabolic, enzymatically controlled activities of the cell are frequently subdivided into two divisions—those involved in the provision of energy and those involved in the synthesis or building up of cellular substance. The synthetic processes, frequently spoken of as *anabolic activities*, are collectively known as *assimilation*, while those reactions involved in the breaking down of matter are known as *catabolic activities*, or *dissimilation*. The dissimilatory reactions can be further subdivided into reactions which yield building material for cellular syntheses and reactions which yield energy for the organism, but this division is hardly worth while since the same foodstuff may serve both as a source of energy and of building material. Present-day concepts in biochemistry suggest that the processes of assimilation and dissimilation are not as distinct from each other as was formerly believed.

The formation and maintenance of the bacterial cell require the provision of a considerable amount of energy. The chemical reactions which yield energy for the cell are collectively known as *respiration* in the broadest sense of the term. No living cell is absolutely still but is undergoing change in one manner or another. The organism may alter its position relative to its environment; it may alter its parts; it may grow; or it may undergo change in constituent molecular structures. This perpetual change of state of the organism requires the expenditure of energy, which must come from some source outside the organism if life is to be main-

TABLE 8-1. IDENTIFICATION TESTS FOR SOME COMMON BACTERIA \*  
(An illustration of their enzymic content)

Organism	Growth		Liquefaction of gelatin	Pigment	Production					Fermentation				
	Aerobic	Anaerobic			Indole	Hydrogen sulfide	Nitrites from nitrates	Acetyl/methylcarbinol	Acid to methyl red	Glucose	Sucrose	Lactose	Maltose	Inulin
<i>Micrococcus pyogenes</i> †														
var. aureus . . . . .	+	+	+	+	—	+	+			A	A	A	A	—
var. albus . . . . .	+	+	+	—	—	+	±			A	A	A	A	—
<i>Streptococcus pyogenes</i> . . . . .	+	+	—	—	—		—			A	A	A		A
<i>Diplococcus pneumoniae</i> . . . . .	+	+	—											
<i>Escherichia coli</i> . . . . .	+	+	—		+	±	+	—	+	AG	AG	AG	AG	
<i>Aerobacter aerogenes</i> . . . . .	+	+	—		±	±	+	+	—	AG	AG	AG	AG	
<i>Proteus vulgaris</i> . . . . .	+	+	+		±	+	+			AG	AG	—	AG	
<i>Proteus mirabilis</i> . . . . .	+	+	+		—	+	+			AG	AG	—	—	
<i>Salmonella typhosa</i> . . . . .	+	+	—		—	+	+			A	—	—	A	
<i>Acetobacter aceti</i> . . . . .	+	—								A	—	—	—	
<i>Bacillus subtilis</i> . . . . .	+	±	+		—	+	+	+		A	A	—	A	
<i>Clostridium butyricum</i> . . . . .	—	+	—		—		—			AG	AG	AG	AG	
<i>Clostridium tetani</i> . . . . .	—	+	+		+		—			—	—	—	—	

\* ±, may or may not be produced or fermented. A represents acid without gas, AG with gas.

† *Staphylococcus aureus* and *albus*.

tained over a long period of time. Considered from a physicochemical point of view, the living cell is a peculiarly constructed energy transformer, through which a continual flux of energy passes, and the entire life of a cell may be looked upon as an expression of alterations or variations in the rate of flow of energy. No artificial system has yet been devised of so complicated a nature as a cell or possessed of such a degree of internal coordination and ability to adapt itself to change. Energy is the underlying cause of all changes in matter, whether animate or inanimate, and therefore we must consider the source of energy required by



the organism, see how it is made available, and how it operates in living matter.

**The Concept of Energy.** At this time it is worth while to attempt to summarize the general concept of energy and of the energetics of chemical reactions before considering the actual reactions involved in the respiration of bacteria. Certain physicochemical concepts, simplified as much as possible, will be introduced to serve as reference material for the discussions on bacterial respiration and growth which will follow. Too frequently, basic considerations of energetics are neglected in discussions of microbial growth and behavior.

Energy may be defined as *the ability of a system to perform work*. It is common knowledge that accomplishment of a task requires the expenditure of energy. The production of light in a lamp bulb requires the expenditure of electrical energy obtained from generators, which in their turn obtained energy for motion from a steam turbine or from a turbine spun by a waterfall, etc. Energy can be converted from one form to another, but in that conversion a portion of the energy is frequently lost as far as doing useful work is concerned. The actual amount of energy converted into work when a definite quantity of energy is available is an indication of the efficiency of the process. Bacteria in general have low conversion efficiencies, only a small (about 10) per cent of the energy stored in a chemical compound actually being employed by the cell for its various activities, the remainder being dissipated as heat.

The cause of all action or change is a result of the tendency for energy to seek a uniform level. Experience has shown that energy does not pass from one system to another at a higher level; e.g., a child soon learns that heat always flows from the hot stove to his fingers rather than in the opposite direction. Because of this tendency of energy to seek a lower level, a molecule of glucose is a good source of energy for the cell, the energy holding the atoms together in the glucose molecule being readily released by cellular enzymes with the production of other molecules of lower energy content. The products of respiration, having a lower free-energy content, are more stable than the material respired, and some of the difference in energy content between the reactants and the products of respiration can be used by the cell in doing work. The cell carries out the reverse reaction, synthesis, only when energy is available, and that energy must come from energy stored in other molecules (or from light in the photosynthetic organisms).

A living cell must perform work of various sorts, and energy must be provided for the performance of this work. All life processes demand for their continuation and maintenance a continuous supply of matter and of energy. As far as matter is concerned, there is only a definite amount of carbon, oxygen, nitrogen, and other substances available to the various



organisms on the earth. Plants and the photosynthetic bacteria feed on terrestrial matter, but energy is supplied to them in the form of light from a source outside the earth. Organic matter synthesized by these forms can serve as a source of building material and energy for other forms of life. These organisms in turn serve as food for still others, but eventually this organic matter is broken down into carbon dioxide and water, which can again enter the cycle of events illustrated in the carbon cycle in Fig 8-1. The fact that plants are able to transform radiant energy into chemical energy was one of the great discoveries in the biological sciences, thus

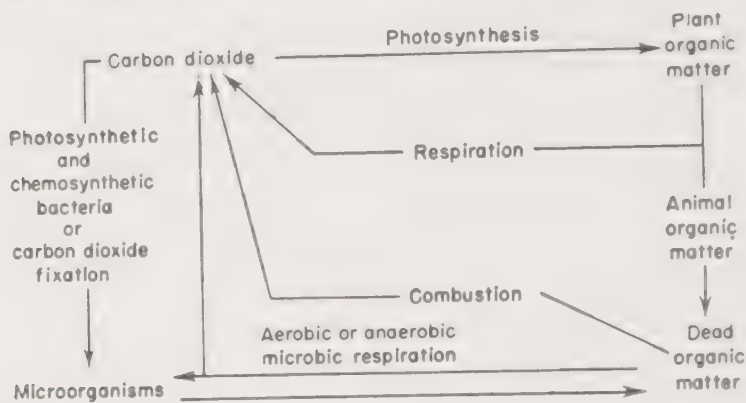


FIG. 8-1. The carbon cycle.

making apparent that the greatest difference between the green plants and the rest of the living world is that the former can obtain the energy necessary for growth and maintenance from light, rather than from matter. On the other hand the nonphotosynthetic cells, or photosynthetic cells in the dark, can transform some of the energy stored up in the chemical bonds of their foodstuffs into the bonds and structures present in those substances entering into the chemical composition of the cell. This appears to be accomplished to a considerable extent with the aid of high-energy phosphate bonds, which will be discussed in the following chapter.

Carbon dioxide and water have relatively low energy contents. When these substances react in the green plant, under the influence of light energy, their bonds are altered and new bonds are produced which hold the atoms together in a different arrangement, e.g., that of the glucose molecule. Light energy has been converted into chemical-bond energy, and these bonds have a high intensity factor as compared with the original bonds in carbon dioxide and water. No change has taken place in the number of carbon and hydrogen atoms in the glucose molecule as compared with the number in the originally reacting carbon dioxide and water molecules, and in fact some oxygen has been lost in the photosyn-

thetic process. Carbon dioxide, water, and light together would not serve as a source of energy and building material for a typical heterotrophic bacterium, but the product of photosynthesis, carbohydrate, together with appropriate salts and water will serve both purposes. The original energy of light stored as chemical energy in the bonds of the glucose molecule is released on oxidation of the glucose, i.e., on the reversal of the synthesis of glucose. Energy so released may undergo conversions within the cell, being utilized in the synthesis of cellular matter, in movement of the cell or of its parts, in the maintenance of the thermodynamically unstable state characteristic of a living cell, and a large portion of the energy is generally wasted in the form of heat. Carbon dioxide and water molecules are reformed, and in the process the original energy of light trapped in photosynthesis has been converted into a variety of forms—chemical, mechanical, electrical, and heat. Heat energy itself is not used to any extent by the cell, the energy of chemical bonds being converted into that of other bonds or to other forms of energy, heat, like carbon dioxide and water, being a waste product of cellular respiration.

We have been stressing that the cell is a converter of energy and at the same time of chemical compounds. The series of conversions might be considered as analogous to those taking place in the generation and use of electrical power. Electricity can be generated by chemical or mechanical means and can be used for a variety of purposes. It can be generated from the kinetic energy of a waterfall; it can be transmitted, stored in storage batteries in the form of chemical-bond energy, and released again when needed, some energy being lost as heat in all the processes involved in the various changes in state of energy. The electrical energy can be employed for the operation of machines used for the production of materials of various sorts, and in all these changes and utilizations we find analogies to events occurring in living matter. No man-devised system, however, is capable of carrying out in one simple unit the multitudinous activities of the living cell.

**Metabolism.** The metabolism of bacteria, as well as that of all other forms of life is concerned with the provision and utilization of energy and of building material. Processes of energy provision and of synthesis are, for the most part, similar or identical in all heterotrophic forms of life and, with the exception of the photosynthetic process, in the autotrophic forms as well. Likewise, cellular constituents are very similar in all forms of life with but few exceptions. Even a superficial survey of the field of biochemistry is apt to fill one with astonishment at the complexity of the chemical constituents of living organisms. This diversity of end products is negligible when one considers the innumerable chemical reactions that are involved in the formation of these products and the fact that directly or indirectly all of them have been derived from carbon

dioxide, water, and inorganic salts. Furthermore, the compounds have to be arranged to form the structural and functional units that comprise a cell. These physiological complexities of life are but suggested in Fig. 8-2.

In so far as the biochemical achievements of the higher animals are concerned, it is possible that metabolism consists to a considerable extent of a rearrangement of the component units of the food, since the necessity for a complex food for this class of organisms is a well-established fact.

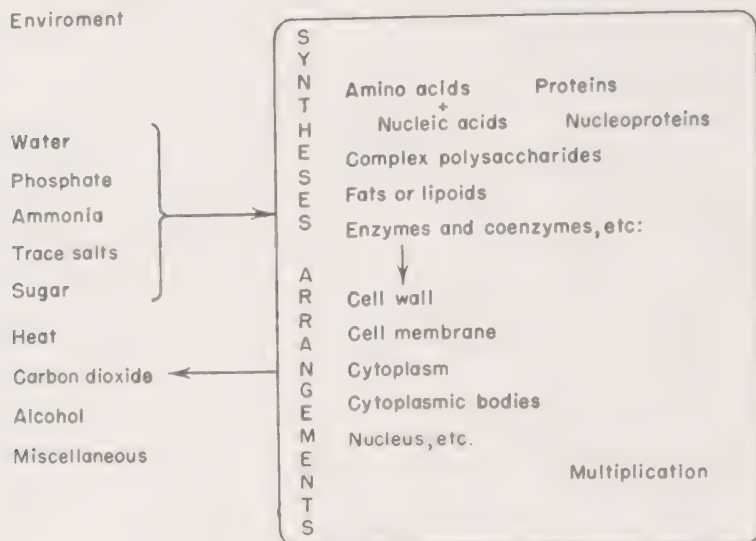


Fig. 8-2. An indication of the complex chemical activities involved in the life of a yeast cell.

But this is not true when we consider the metabolism of the saprophytic microorganisms, for it has been known since the time of Pasteur that a single organic compound, e.g., tartaric acid, in a saline solution may suffice to support growth of these organisms. Also, the organic compound serving as a source of carbon for synthesis and as a source of energy may be replaced by one of a hundred or more different organic compounds, yet the chemical complexity of the bacterial cells so formed may be no less than that of the cells of higher organisms. Still we speak of them as simple organisms! Ascending the scale of bacterial evolution, we find that the food requirements may approach in complexity the requirements of the higher forms of life. Therefore an understanding of the metabolic activities of bacteria is of extreme value in elucidating similar activities carried out by higher forms of life. Furthermore, the study of the metabolism of bacteria offers two advantages over that of the higher organisms in that (1) it is possible to define clearly the initial and final

states of the system which undergoes chemical conversion and (2) the bacteria are rather uniform catalytic agents.

If an attempt is made to survey the mass of observations concerning the chemical changes to which the food is subjected under the influence of bacteria, it is found that complex polymers are hydrolyzed into their molecular constituents, e.g., starch to sugar. While these hydrolytic processes are remarkable, many of them can be reproduced in vitro with the aid of inorganic catalysts. Since hydrolysis is involved only in breaking bonds between carbon and oxygen or nitrogen atoms in the molecule and provides little or no energy for cellular activities, energy must be obtained from other reactions in which the carbon skeleton is greatly modified and bonds between carbon atoms are broken or formed.

A closer consideration of metabolism shows that the bulk of the components of foodstuff are never integrally converted into cellular constituents. On the contrary, a large portion of the food leaves the cell again after having undergone marked chemical change. This latter phase of metabolism, catabolism, from which most of the energy available to the cell arises, lends itself more readily to study than do the anabolic activities of the cell. Dissimilation consists primarily of enzymatically catalyzed oxidations of the substrate, and it is therefore of importance to obtain a clearer insight into the mechanism of the catalytic reactions involved in the utilization of foodstuffs by bacteria, and incidentally by other forms of life.

**Biological Oxidations.** The great majority of bacteria are chemosynthetic. The energy they require for synthetic purposes is obtained by oxidation reactions from the energy stored up in chemical compounds. Except in the case of the autotrophic forms, the oxidizable, energy-providing foodstuffs are organic compounds. The catabolic activities make possible a continuous, regulated supply of energy to the cell. These activities are grouped under the term *respiration*, which in its broadest sense can be considered as *the sum total of the chemical reactions from which energy is made available to the cell*.

Originally, biological oxidations were assumed to be the oxidation of organic matter by molecular oxygen, carbon dioxide and water being recognized as end products of oxidation. Studies on the chemistry of oxidation reactions suggested that in the oxidation of ferrous oxide to ferric oxide, while it is true that there is an uptake of oxygen ( $2\text{FeO} + 0.5\text{O}_2 \rightarrow \text{Fe}_2\text{O}_3$ ), the fundamental change is one of valence ( $\text{Fe}^{++} \rightarrow \text{Fe}^{+++}$ ), since conversions such as ferrous to ferric chloride involve the same change as far as the iron atom is concerned. Oxidation in its broadest sense is now defined as *the loss of one or more electrons*. An equivalent amount of reduction must accompany any oxidation process, and reduction is defined as the gain of one or more electrons.



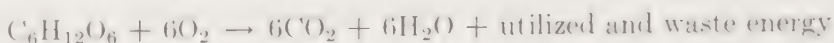
With bacteria oxidizing hydrogen or carbon monoxide as their source of energy, we can write



and



These reactions indicate an uptake of gaseous oxygen by the substances being oxidized, oxygen being reduced as a result of the reactions. In most aerobic biological oxidations, oxygen from the air is not added to the substance undergoing combustion but instead hydrogen and electrons are withdrawn from the substrate and combine with the oxygen taken up by the cells. The over-all reaction for the oxidation of glucose can be expressed as



The three oxidations depicted above proceed with the liberation of chemical-bond energy, a portion of which may be used by the cells, the remainder dissipated as heat. Bacteria and other microorganisms which grow in the presence of air and which consume oxygen are spoken of as *aerobes*, or *aerobic organisms*. Other species, *anaerobes*, can grow in the absence of oxygen and use substances other than oxygen as the oxidizing agent.

Bacterial or other cells, including those of our body, cannot tolerate or utilize the heat given off when a substance is oxidized by direct combustion. Instead they utilize mechanisms by means of which the foodstuff is broken down in a series of reactions during which a portion of the energy of oxidation is retained in new chemical bonds, the excess being wasted in the form of heat energy. Energy is trapped primarily from oxidations and in the form of so-called high-energy phosphate bonds which can be transferred from the material undergoing oxidation to adenosine diphosphate (ADP) with the formation of adenosine triphosphate (ATP, see Fig. 8-3). ATP is the cell's storehouse of energy and, by taking part in coupled reactions, it can transfer energy to other molecules, thus making them more reactive. ADP is formed in the exchange, and thus the ATP-ADP system acts in a cyclic manner, analogous to a storage battery which can be alternately charged and discharged. By taking part in a wide variety of reactions, ATP is able to transfer energy from energy-providing (exergonic) reactions to energy-requiring (endergonic) ones, thereby enabling the cells to synthesize cellular substance and to carry out their various functions.

The formation and transfer of high-energy or energy-rich phosphate bonds can be illustrated if we consider the simple case of the oxidation

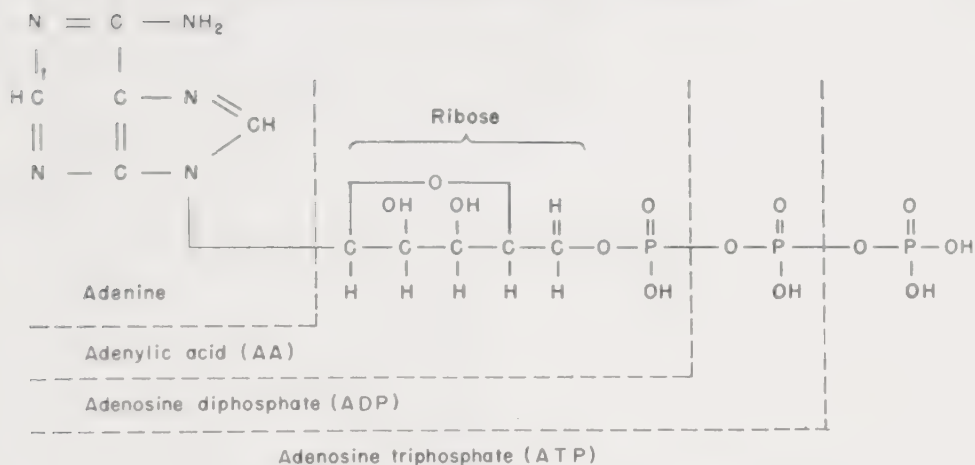
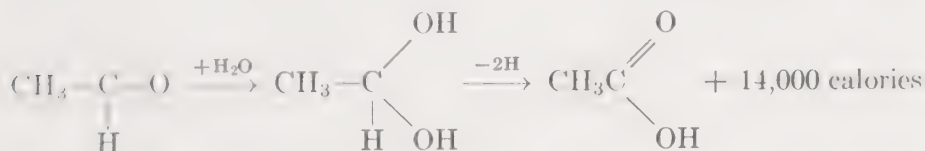
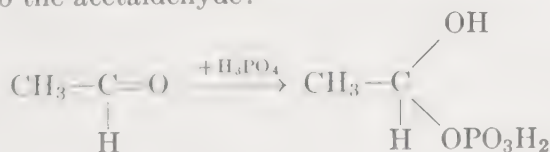


Fig. 8-3. The adenosine triphosphate coenzyme of phosphorylation.

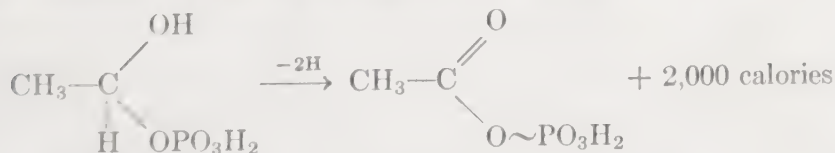
of acetaldehyde to acetic acid. If this reaction took place in the test tube we would find that water is added to the aldehyde molecule, which is then oxidized by loss of hydrogen (to oxygen), or



In the cell, however, an enzyme catalyzes the addition of phosphate rather than of water to the acetaldehyde:



and on oxidation by loss of hydrogen, acetyl phosphate is formed:



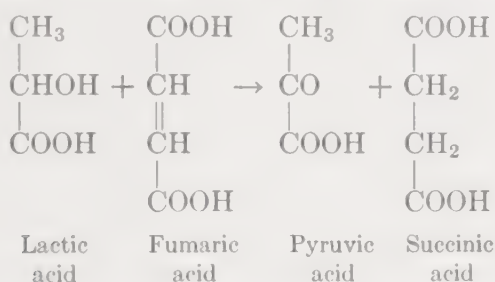
the high-energy bond being represented by the wavy line  $\sim$ . Hydrolysis of the acetyl phosphate to give acetic acid and phosphate would yield around 12,000 calories, thus indicating that this amount of energy is associated with the high-energy phosphate bond. Or, if acetyl phosphate reacted with ADP to form acetic acid and ATP, about the same amount of energy would be released, as indicated in the above equation,

while that associated with the energy-rich bond would be retained in the ATP molecule. In this manner energy is trapped and stored in the cell. Energy-rich compounds can also be used directly for synthetic purposes, e.g., acetyl phosphate can react with a substance such as oxalacetic acid to form citric acid and phosphate, the energy required for this synthesis coming from that associated with the high-energy phosphate bond. The peculiar nature of this bond provides a connecting link between anabolic and catabolic activities in the cell and is involved in both aerobic and anaerobic life.

**Anaerobic Life.** In 1861 Pasteur reported that there are bacteria capable of growing, and hence respiring, in the complete absence of air (oxygen). At that time it was believed that air was essential for life, but with the discovery of "life without air," Pasteur became interested in the mechanism by which these organisms obtained the energy to maintain their life processes. He proved that yeasts convert glucose to carbon dioxide and ethyl alcohol, while certain species of bacteria convert this sugar to lactic acid or to butyric acid. None of these products were of any apparent benefit to the microbes, and in fact their accumulation led to the inhibition or death of the cells. The term fermentation ("to boil") was originally employed to denote the reaction by which alcohol and carbon dioxide are formed from sugar but was broadened by Pasteur to include the various decompositions of sugar brought about by microorganisms in the absence of air. While the term fermentation is generally limited to the decomposition of carbohydrates, it may also be applied to the breakdown of proteinaceous material, the mechanism of the reactions being essentially similar. Ordinarily the decomposition of protein in the absence of air is spoken of as *putrefaction*. (The term *decay* is used to represent the decomposition of organic matter in general, particularly under aerobic conditions.) What part does fermentation play in the life processes of the *strict anaerobes* or in those cells, the *facultative anaerobes*, which can live either in the presence or the absence of oxygen?

In time it became apparent that the formation of ethyl alcohol and carbon dioxide, of lactic or butyric acids, or of other products of fermentation involved oxidation-reduction reactions in which molecular oxygen was not involved. Biological oxidations, aerobic or anaerobic, can in many instances be interpreted on the basis of electron exchange but in general are more easily considered as transfers of hydrogen. Molecular oxygen is commonly reduced by cells growing under aerobic conditions. It, however, a cell is able to reduce substances other than oxygen, the energy-liberating oxidations may proceed in the complete absence of air. Early in the study of anaerobic respiration it was observed that nitrates could serve as an oxidizing agent, being reduced to nitrites. The oxygen liberated in the nitrate-nitrite conversion was assumed to replace molec-

ular oxygen. In other cases, however, the oxidizing agent did not liberate oxygen on reduction but instead took up hydrogen, e.g.,

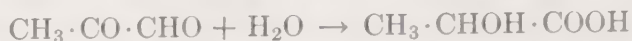


Lactic acid is oxidized in this reaction by loss of hydrogen, which is taken up by fumaric acid, the latter being reduced to succinic acid. If the same oxidation took place under aerobic conditions, lactic acid would also be oxidized to pyruvic acid by loss of hydrogen, the hydrogen being taken up by oxygen, which would be reduced to water. We must bear in mind that fermentations involve oxidations of foodstuffs just as much as oxidations involving the uptake of oxygen, the difference being in the ultimate oxidizing agent, oxygen or other matter.

Compounds which give up hydrogen are known as *hydrogen donors*, while those which receive it are known as *hydrogen acceptors*. Some dyes (e.g., methylene blue) and certain naturally occurring substances (such as limus and nitrates), may accept hydrogen when in the oxidized state and thus be reduced; then they in turn may donate this hydrogen to another acceptor. Those compounds which can act as reversible oxidation-reduction systems are known as *hydrogen carriers* and, when present in the cell, work in conjunction with the enzymes to facilitate oxidation by transporting the hydrogen from the foodstuff to a suitable hydrogen acceptor. They are an example of one group of agents spoken of as *co-enzymes*, as they aid or implement the action of an enzyme. Methylene blue can act as an artificial hydrogen carrier in test-tube (in vitro) studies. If a suspension of bacteria is allowed to stand in the presence of a foodstuff and methylene blue, the dyestuff will become colorless, methylene blue being blue in the oxidized state and colorless in the reduced state (leuco base). When the tube is agitated, oxygen will penetrate into the fluid, and the reduced methylene blue will be reoxidized, hydrogen being donated to the molecular oxygen. The methylene blue will then be in a state where it can once more accept hydrogen and again be reduced. Thus its carrier action can be visually demonstrated. Biological oxidations can generally be considered as transfers of hydrogen, the hydrogen from the substrate ultimately uniting with oxygen under aerobic conditions, or with some other hydrogen acceptor under anaerobic conditions. Electrons are also transferred in the oxidation, a fact that is



generally inferred but not always expressed in the consideration of biological oxidation. These reactions are for the most part *intermolecular* in character, but in some instances *intramolecular* oxidation-reduction reactions may occur. In the latter type of respiration, one portion of the molecule is oxidized while a second portion is reduced. For example, methylglyoxal, after taking up a molecule of water, may undergo intramolecular oxidation-reduction with the formation of lactic acid:



the central carbon atom being reduced, the terminal or aldehyde carbon atom being oxidized.

To recapitulate, we find three general types of biological oxidations, namely, hydrogen from the foodstuff being accepted (1) by oxygen, (2) by another molecule—either the foodstuff, decomposition products of the foodstuff, or an entirely different molecule (other than oxygen) which may be organic or inorganic in character—or (3) by a different portion of the same molecule. Oxidation of the foodstuff need not be complete, even in the presence of oxygen. For example, some bacteria oxidize ethyl alcohol to carbon dioxide and water while others (the vinegar bacteria) oxidize it only to acetic acid. Glucose is oxidized to completion by many organisms, i.e., to carbon dioxide and water, while other species oxidize it only as far as oxalic or gluconic acid. The extent of the oxidation, in the presence of an excess of oxygen, is controlled by the enzymic structure of the cells. Partial oxidation and anaerobic life are in general more wasteful of foodstuff than complete aerobic life. When the foodstuff is oxidized to completion, more energy is available to the cell, 686,000 cal. of free energy being liberated per mole of glucose completely oxidized, while if fermentation occurs with the production of lactic acid, approximately 36,000 cal. is available. For an equal amount of growth, approximately 19 times as much glucose would have to be consumed under anaerobic as under aerobic conditions, provided that the efficiency of assimilation is the same under both anaerobic and aerobic conditions.

Many microorganisms are neither strict aerobes nor strict anaerobes but will grow under either condition in a suitable medium. Generally growth of these *facultative anaerobes* is more rapid and occurs to a greater extent under aerobic than under anaerobic conditions. A few species grow best under semiaerobic conditions, i.e., under a lower oxygen tension than when exposed to air, but some oxygen must be provided for the cells. These are spoken of as *microaerophiles*.

**Nutritional Requirements of Bacteria.** A consideration of the sources from which bacteria obtain their energy and building materials enables us to classify the bacteria on the basis of their nutritional requirements.

In our survey of microbial life certain important differences were noted in the modes of nutrition of the various groups, and amongst the bacteria we find equally divergent energy and building-material requirements. Knight has divided bacteria into four main groups on the basis of their general nutritional requirements, a classification which has been considered by some to represent nutritional evolutionary stages in the development of bacteria. These four groups, with slight modification, can be briefly summarized as follows:

*Group 1.* Carbon is assimilated as carbon dioxide and nitrogen from inorganic sources, generally ammonia. The energy required for the reduction of carbon dioxide to organic matter is derived (1) from the oxidation of inorganic matter by the *chemosynthetic autotrophs* or (2) from radiant energy (light) by the *photosynthetic autotrophs* and the *photosynthetic heterotrophs*.

*Group 2.* Carbon is assimilated from organic matter and nitrogen from inorganic sources, generally ammonia. Energy is obtained from the oxidation of organic matter. These bacteria might be designated as *non-extracting chemosynthetic heterotrophs*.

*Group 3.* Carbon is assimilated from organic matter, but ammonia does not serve as the sole source of nitrogen, specific amino acids frequently being required. *Semiextracting chemosynthetic heterotrophs*.

*Group 4.* Carbon is assimilated from organic matter while nitrogen requirements generally include more than one amino acid. Accessory growth-promoting factors—vitamins—are also required. These cells are frequently parasitic and can be designated as *extracting heterotrophs*, or *facultative parasites*.

To these four groups a fifth group might be added to include the rickettsiae and the filtrable viruses. This group could be defined as follows:

*Group 5.* Carbon and nitrogen assimilated only from and with the help of other living and generally highly specific cells. *Obligatory heterotrophic parasites*.

It can be seen that there is a progressive increase in the nutritional requirements from group 1 through group 4 (or 5), as regards first the source of carbon and second and probably most important, the source of nitrogen. In the first group there are organisms which can grow in an entirely inorganic medium (if carbon dioxide and the salts of carbonic acid are considered inorganic substances), some obtaining their energy from the oxidation of inorganic matter while others utilize light energy. The photosynthetic heterotrophs might be considered as a borderline group, but the main function of the organic matter appears to be as a source of hydrogen for the photosynthetic assimilation (reduction) of carbon dioxide.

The second group comprises forms transitional between strict autotrophs and strict heterotrophs. The third group requires more strictly heterotrophic conditions, while the fourth and fifth groups, comprised of the stricter parasites, are very exacting in their nutritional requirements. Also, with increase in nutritional requirements, there is a tendency for a progressive increase in parasitism and pathogenicity to occur in the groups 3 to 5.

Such a classification is by no means complete, and there are no sharp lines of demarcation between the groups. Furthermore there are organisms, (e.g., certain of the photosynthetic bacteria, *Athiorhodaceae*, in group 1) which do require specific growth factors but for all other purposes fall into a group other than 4. The classification therefore primarily shows trends rather than absolute characteristics. Also, present-day studies in biochemistry indicate that while these organisms differ markedly in their general nutritional requirements, many, if not the majority, of the assimilatory and dissimilatory reactions involved in other than the primary stages of synthesis are similar or identical in the various groups, as well as in other forms of plant and animal life.

An organism capable of utilizing a purely inorganic diet—salts, carbon dioxide (or carbonic acid or its salts), and water—is entirely independent of other forms of life for its existence, as long as these substances are available in assimilable form. The cell is said to be self-sufficient—*autotrophic*—in its nutrition. The plants are autotrophic in their mode of nutrition, obtaining the energy necessary for the reduction of carbon dioxide to organic matter from light and the hydrogen for this reduction from water. The net result of photosynthesis in the green plant can be represented as

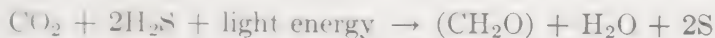


the product of photosynthesis having the empirical composition of a carbohydrate. But apparently in the green plant, and in other autotrophic organisms, once the essential units of organic matter for building purposes have been synthesized, the internal metabolism of the cell from then on is no longer autotrophic. The autotrophic cells do not in general appear to be able to utilize organic matter *from their environment*, but they do utilize organic matter that is formed *within* their cells.

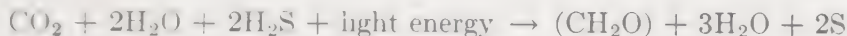
The green plant reduces carbon dioxide with hydrogen derived from the photolytic splitting of water into hydrogen and hydroxyl radicals. The hydroxyl radicals are disposed of as water and oxygen. The photosynthetic autotrophic bacteria do not possess the enzymes required for this latter reaction and instead use the oxidizing hydroxyl radical for the oxidation of appropriate inorganic compounds, e.g., hydrogen sulfide to sulfur or sulfate. It was formerly believed that the hydrogen for the



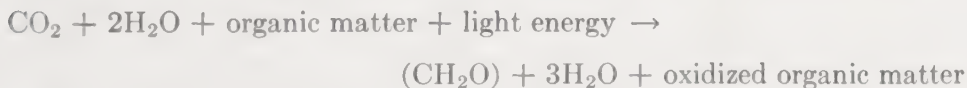
reduction of carbon dioxide came from the inorganic substrate, since the over-all reaction can be expressed as



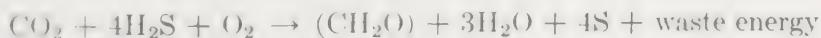
The more accurate expression would be



Note that the photosynthetic bacteria do not produce oxygen as a waste product as do the green plants and instead, in the above example, sulfur is the waste product of photosynthesis. The photosynthetic heterotrophs carry out a similar reduction of carbon dioxide, with the exception that the final reducing agent is an organic compound instead of an inorganic one. Photosynthesis by heterotrophic bacteria can be represented as



The nonphotosynthetic autotrophs also reduce carbon dioxide to assimilable organic matter but, lacking the required photosynthetic pigments, must obtain their energy from the oxidation of inorganic matter such as sulfur (or compounds thereof), iron salts, ammonia, nitrites, or hydrogen. Apparently the energy available from the oxidation of the inorganic matter takes the place of the light energy absorbed by the photosynthetic pigments. A typical autotrophic chemosynthetic reduction of carbon dioxide may be represented as



Actually none of these reactions are as simple as written, but they do picture the basic assimilatory reaction. These autotrophic organisms are highly specific as regards source of energy; e.g., the sulfur-oxidizing bacteria are unable to oxidize iron salts, ammonia, or hydrogen as a source of energy.

Organisms on the border line between group 1 and group 2 obtain the energy required for the reduction of carbon dioxide from the oxidation of carbon compounds such as carbon monoxide or methane. Finally we find in group 2 and in the remaining groups, organisms—the heterotrophs—which can take up organic matter from their environment and obtain from it by oxidation processes the energy required for the synthesis of cellular matter, at the same time utilizing a portion of this organic matter for building purposes. It should be mentioned that some carbon dioxide may be assimilated by all microorganisms or by higher forms of life, but it does not constitute the main bulk of the assimilated carbon. The bacteria in groups 2 to 4 (and also the yeasts and molds)



are *saprophytic* in their nutrition, absorbing through the cell membrane organic matter dissolved in water. In time, certain of these saprophytic organisms may have become adapted to life on or in other living matter, and thus we find that they developed to a greater or lesser extent the property of *parasitism*. When these parasitic microorganisms produce marked damage in their host's tissues, they are said to be *pathogenic* and are spoken of as *pathogens*. In the course of adaption to the parasitic mode of life, we frequently find that the parasite adapts itself still further to its host by becoming highly dependent upon it for the supply of one or more essential components of the parasite; i.e., with the development of parasitism there generally appears to be a concurrent decrease in the powers of synthesis possessed by the parasitic organism.

**Growth and Respiration in the Test Tube.** To illustrate growth-respiration relationships under the conditions prevailing in test-tube cultures, let us consider what occurs when *Escherichia coli* is inoculated into a solution of inorganic salts with glucose as the organic food-stuff. Some of the glucose will be assimilated, some will be oxidized to carbon dioxide and water, but as the bacterial population increases, the oxygen dissolved in the medium will be consumed. Since the rate of diffusion of oxygen from the air into the medium is very slow, aerobic conditions will no longer exist in the depths of the medium. The bacteria must then obtain energy by means of anaerobic oxidations (fermentations), and products such as lactic, acetic, and succinic acids will be formed from the glucose and accumulate in the medium. Growth in the depths of the medium will cease when all the glucose has disappeared but may be re-initiated to a slight extent as oxygen diffuses into the medium, the organic acids then being used as a source of carbon compounds and also being oxidized, with the participation of oxygen, to carbon dioxide and water. They can be oxidized under aerobic conditions and will support growth, since oxygen will accept hydrogen from them, while under anaerobic conditions no suitable hydrogen acceptor is available for the oxidation of the acids produced from the glucose. If the tube was not disturbed during the course of the experiment, it might be observed that growth occurred more readily in the upper portion of the medium, since conditions there are generally more aerobic than in the butt of the tube. Thus the bacteria in an ordinary test-tube culture in a liquid medium are growing under all conditions of aerobiosis, the oxygen gradient ranging from highly aerobic to practically, if not completely, anaerobic conditions. It should be mentioned that aerobic conditions are generally toxic to the true anaerobes, no completely satisfactory explanation for this fact being known. In many instances the accumulation of hydrogen peroxide may be the main inhibitory factor, the anaerobes lacking the enzyme catalase.

involved in the disposal of hydrogen peroxide as water and oxygen, but this explanation is not entirely satisfactory.

Cultures growing on a solid medium such as nutrient agar generally have free access to oxygen at first, but anaerobic conditions can develop within the mass of growth, since oxygen may be utilized before it diffuses into the bulk of the colony. Also the carbon dioxide liberated in the respiratory process tends to prevent the inward diffusion of oxygen.

**Biochemical Activities of Bacteria.** The nutritional requirements of bacteria, as outlined earlier in the chapter, vary between different species and range from inorganic salts through simple organic compounds to the more complex ones such as cellulose and proteins, or even the tissues of a host. From the diet suitable for the multiplication of a particular species of bacteria, a single cell is able to synthesize sufficient cellular matter to enable it to reproduce itself in a short period of time, generally a matter of minutes under favorable conditions. All these synthetic reactions are catalyzed by the enzymes present in and characteristic of the particular species. As yet, these anabolic activities of the cell are difficult or impossible to present in simple chemical equations, although considerable progress is being made in the study of the synthesis of various cellular constituents. Not only does the diet of bacteria vary with the species, but also there is wide variation in the types of reactions involved in the dissimilatory reactions which provide some of the building material and also the energy required for the synthesis of cellular matter and for the maintenance of life.

Very few bacteria always digest their food in the same way as animals do, i.e., primarily by complete oxidation of the digestible matter to carbon dioxide and water. Most of the strict aerobes and facultative anaerobes can carry out a complete combustion of the carbonaceous matter not entering into cell structure, but as a rule, both in nature and in the test tube, the supply of oxygen for this purpose is limited by its low solubility in water. Rahn<sup>1</sup> has pointed out that in the manufacture of tuberculin, the tubercle bacilli are grown in a medium containing 3 per cent glycerol as the oxidizable material for the supply of energy. To oxidize the amount of glycerol in a quart of medium, approximately 3 oz. of oxygen are required; yet the solubility of oxygen is only about 0.0003 oz. per quart of medium. Any interference with the free diffusion of oxygen into the medium could retard the growth of the tubercle bacillus. Similar considerations hold for any bacteria growing under aerobic conditions. Conditions in nature are frequently not so suitable for the growth of bacteria as those in the test tube, and during the course of time many species have become adapted to growth under diminished oxygen supply

<sup>1</sup> Otto Rahn, "Microbes of Merit," The Ronald Press Company, New York, 1945.

or in the complete absence of oxygen. The digestions of organic matter which they carry out under anaerobic conditions are not complete, and the waste products of these bacterial fermentations are often characteristic of a particular species. The lactic acid bacteria of milk characteristically partially digest (ferment) sugars with the production of lactic acid; the propionic acid bacteria active in the production of Swiss cheese produce propionic and acetic acids and carbon dioxide (responsible for the holes); while the coliform bacteria produce an even greater number of end products of the fermentation of sugar. Similarly, proteins can be digested in the absence of oxygen with the production of a variety of end products, some of which are putrid-smelling compounds and hence gave rise to the term putrefaction for the anaerobic fermentation of proteinaceous matter.

Many species of bacteria, the facultative anaerobes, are able to grow under either aerobic or anaerobic conditions. Under aerobic conditions they commonly oxidize the organic foodstuff to completion; under anaerobic conditions it is only partially digested, and many of the products of partial digestion are valuable to man in his everyday life. They are also of value to the bacteriologist, since he commonly tests for the production of characteristic end products of the fermentation of sugars and of amino acids as a helpful procedure in the identification of many species of bacteria. These digestions, like the syntheses previously mentioned, are controlled by the enzyme pattern of the species. It should be reemphasized that both aerobic and anaerobic respiration involve primarily splitting of the foodstuff molecules into simpler units and oxidation of these units. Oxidation under aerobic conditions results in the transfer of hydrogen and electrons to oxygen; under anaerobic conditions the transfer is to organic molecules formed during the course of degradation of the foodstuff or at times to other hydrogen acceptors such as nitrates or sulfates. Since these hydrogen acceptors are generally limited in amounts present in the medium or in amounts formed during dissimilation, oxidation cannot proceed to completion since the available hydrogen acceptors limit the extent of the oxidation. Other, more complex factors also play a role in preventing oxidation to completion under anaerobic conditions.

Observations on both the particular foods utilized and the end products of their utilization are employed in the identification of different species of bacteria as is illustrated in Table 8-1. The bacteriologist commonly tests for the ability of an unknown organism to ferment various sugars, as evidenced by acid or acid and gas production, or to hydrolyze complex carbohydrates such as starch or cellulose (or even agar) into simpler, fermentable sugars; for the production of hydrogen sulfide from sulfur-containing amino acids; for the liquefaction of gelatin or of coagulated



serum; for the coagulation of the casein in milk; for the reduction of dyestuffs such as methylene blue or litmus or the reduction of nitrates; for the production of indole from the amino acid tryptophan or of ammonia from amino acids in general; and to a lesser extent for the production of other specific products such as acetylmethylcarbinol. The beginning student tends to think of these tests primarily as diagnostic procedures, while actually the results of these tests reflect the enzymic content and activities of the species tested. Types of colonies produced, characteristics of growth in liquid media, pigment formation, ability to produce an infection in plants or animals, and other biochemical activities (or activities ultimately based thereon) are also employed in the identification of an unknown species, using reference keys to the bacteria based upon such characteristics. The keys most widely used for such a purpose in this country are presented in "Bergey's Manual of Determinative Bacteriology." All the biochemical tests reflect the enzyme pattern characteristic of a species, a pattern which is subject to variation within limits controlled by the genetic pattern of the species and by those environmental factors which influence the activity of enzymes. Actually the enzyme pattern is controlled by the genes; the metabolic activities of a cell reflect its enzyme pattern, these enzymes in turn being produced under the pattern established by the genes characteristic of a given species.

Since a complete understanding of bacteria and their behavior necessitates a fuller understanding of the metabolic activities of bacteria, these biochemical activities are considered in more detail in the two following chapters. The more general aspects of metabolism are considered in Chap. 9 while Chap. 10 is devoted to the metabolism of individual groups of bacteria. Much of the material presented in these chapters is of a somewhat complex biochemical nature but is simplified in so far as consistent with the development of a reasonably complete picture of the metabolic activities of the bacteria. The detailed biochemical considerations are intended primarily as reference material, to illustrate the complex but carefully coordinated metabolic activities of bacteria and other cells. An attempt is made in Chap. 9 to develop a simple picture of metabolism before proceeding with the more complex aspects. Final choice of material to be covered can rest with the instructor as he sees fit for the development of the course.

## REFERENCE

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## CHAPTER 9

### MECHANISMS OF MICROBIAL RESPIRATION

The study of bacteria as physiological agents may be said to have had its origin in Pasteur's work on fermentation and its early development in the hands of Winogradsky, Omeliansky, Beijerinck, Harden, and others. The majority of the applications of chemistry to microbial activity were made in those fields where the chemical changes produced by the microbes were fairly well defined and led to the production of substances of economic importance. In more recent years the tendency has been to study the various microorganisms both as living agents and in an attempt to obtain a more complete understanding of their varied and numerous activities in the soil, in water, in their industrial applications, and in their relation to infectious diseases in plants and animals. Bacteria and yeast, owing to their relatively small size, simplicity of organization, rapid growth, and ability to adapt themselves to changes in their environment, make excellent test agents for the study of many chemical reactions of physiological importance, and many of the advances in biochemistry in recent years are due in part to studies with these organisms.

To form any completely coherent picture of the essential chemical processes accompanying the life of these minute organisms is as yet beyond our power. But much is known about their general metabolic activities, and we can attempt to consolidate our knowledge of the metabolism of microorganisms, whether they are agents of disease or bearers of economically important enzymes. From the biochemical point of view, the fact that a particular microbe will grow only in a complex medium is not of importance except in so far as it brings forth the questions as to what the chemical substances are in the medium that are conducive to growth, what their functions are, and why one organism, but not another, requires these particular substances. From the same viewpoint the lesions produced in infectious diseases are not of so much interest as the microbial processes, and their interplay with the host, which lead to the typical disturbances in the host's tissues. Thus the problems become more complex and require that interest be developed in the *how* and *why* of microbial activity. We have already summarized the *why* of microbial activity as the necessity of the cell to obtain energy and

building material for its maintenance and growth. Let us now turn to a consideration of *how* microbes, and incidentally other forms of life as well, carry out these processes.

We now know that microorganisms are responsible for decay, for fermentation, for the maintenance of soil fertility, for the production of infectious diseases, and for numerous other activities, but it was only about one hundred and twenty years ago that Thenard, and a little later Schwann, proposed that fermentation is brought about by the activities of yeast cells. Before this proposal it was believed that fermentation was a natural decomposition of sugar in fruit juices and that fermentation provided conditions conducive to the growth of microorganisms, rather than being caused by them. The famous German chemist Liebig maintained that fermentation is a spontaneous process induced by unstable molecules in the fluid and that microorganisms may appear in the fermenting liquid since they are simply bits of loosely organized organic matter. Schwann's concept of fermentation gave rise to a bitter controversy between his followers and those of Liebig, and the controversy is well illustrated by an anonymous satirical article which appeared in *Justus Liebig's Annalen der Chemie* in 1839. Quoting in part from a translation by E. F. Kohman:<sup>1</sup>

Beer yeast, dispersed with water, is dissolved under this instrument [described as a remarkable new microscope] into endless small pellets whose diameter is scarcely  $\frac{1}{500}$  that of a line, and into fine threads which are unmistakably a sort of protein material. If one introduces these pellets into sugar water, one observes that they consist of the eggs of an animal; they swell, burst and tiny animals are evolved from them, which multiply with inconceivable rapidity in a most unparalleled manner. The form of these animals deviates from every one of the 600 hitherto described species; they possess the shape of a tiny "Beindorf" distillation flask (without the cooling apparatus). The nozzle of a helmet is a sort of sucking proboscis which is lined on the inside with bristles  $\frac{1}{2000}$  of a line in length; teeth and eyes are not noticeable; on the other hand, one can easily distinguish a stomach, intestinal canal, the anus (as a rose-colored point) and the urinary secretory organs. From the moment that they have escaped from the eggs, it is observed that these animals gobble up the sugar out of the solution; very plainly it is seen as it arrives in the stomach. Momentarily it is digested, and this digestion is simultaneous with and in a most concise manner distinguishable from the evacuation of excrement which follows. In a word these infusoria eat sugar, evacuate from the intestinal tract alcohol, and from the urinary organs carbon dioxide. . . .

Quantitative data, as well as drawings of the form of these animals are to follow in a more detailed treatise.

Needless to say the detailed treatise never appeared, but this article does indicate the extent of the controversy.

<sup>1</sup> *Journal of Chemical Education*, **10**, 543 (1933).

Lavoisier in 1789 had demonstrated that alcoholic fermentation consisted chemically of a decomposition of sugar into ethyl alcohol and carbon dioxide, the quantitative aspects of alcoholic fermentation being established in 1815 by Gay-Lussac, who reported that the chemical reaction may be expressed as



Berzelius around 1837 recognized the similarity between the fermentation of sugar and the decomposition of molecules such as those of hydrogen peroxide by catalysts. He spoke of fermentations as catalytic life processes, or the results of the catalytic power of tissues, but was unwilling to ascribe alcoholic fermentation to the activities of minute, self-reproducing yeast cells. Berzelius pictured alcoholic fermentation as being caused by yeast, which he considered to be a *ferment* produced by the spontaneous oxidation of grape juice. His concept was strengthened by the fact that the ferments (enzymes) diastase, emulsin, pepsin, and trypsin had been obtained free from living cells and that these agents were able by themselves to bring about the catalytic decomposition (hydrolysis) of starch, polysaccharides, and nitrogenous matter, respectively. Remember that at this time the cellular concept was just being established and that little was known concerning the nature of microorganisms.

Pasteur published several papers around 1860 to 1879 in which he pointed out that when fermentations occur, certain microorganisms abound in the liquid; that when these organisms are introduced into an unfermented liquid, the same kind of fermentation is induced; and that the nature of the fermentation depends upon the kind of microorganism introduced into the medium. Finally he pointed out that when these microorganisms are excluded, no fermentation occurs. According to Pasteur's concept, alcoholic and other fermentations are closely connected with the life of living cells, which he considered as *organized* or *formed ferments*, while diastase, emulsin, pepsin, and trypsin are *unorganized ferments* since they do not require the presence of living cells for their activity. In 1878 Kühne proposed the name *enzyme* for this latter group of ferments. Those who believed in vital forces claimed that marked differences existed between fermentations and enzymatic reactions, the former occurring only in immediate contact with the living cell, while enzymes acted when separated from the cell.

In 1897 Buchner demonstrated that fermentation can occur in the absence of yeast cells. On crushing yeast with sand and filtering off the yeast juice under pressure, he obtained a cell-free preparation capable of converting sugar to ethyl alcohol and carbon dioxide. This discovery led to the concept that, although protoplasm is the producer and carrier of enzymes, yet protoplasm can often be broken down without injury to the



enzymes. The difference between ferment and enzyme then disappears, and we now recognize that enzymes are formed by the cell but are not necessarily dependent upon the cell for their activity.

Enzymes are loosely defined as organic catalysts of biological origin. They may also be defined in functional terms as *biocatalysts of diverse chemical nature which by chains of linked reactions implement biological oxidations, hydrolyses, syntheses, and other reactions, at the same time imparting order and direction to these reactions within the living cell.*

**Catalysis.** Since we have defined enzymes as biocatalysts, we should first consider what is meant by the terms catalyst and catalysis, as our definition implies that enzymes are catalysts of biological origin. A catalyst is defined as a substance which changes the rate of a chemical reaction without itself being used up in the reaction, and such alteration in reaction velocity is called catalytic action, or catalysis, the word catalysis meaning "loosening down." Most catalysts accelerate reactions, but examples of negative catalysis are known. A chemical reaction may occur spontaneously if there is a decrease in free energy as a result of the reaction, but, although the reaction *may* theoretically be possible, we must remember that the reaction does not need to occur at an appreciable rate. Foodstuffs can be oxidized to carbon dioxide and water spontaneously in the presence of oxygen, but this oxidation does not occur at an appreciable rate with ordinary foods and at low temperatures. (Note the fact that many of the foodstuffs buried with the Egyptian Pharaohs were still intact when the tombs were opened after thousands of years.) In the presence of the appropriate biocatalysts the same foodstuffs would be oxidized rapidly.

Hydrogen peroxide is fairly stable in dilute solution but decomposes rapidly into water and oxygen in the presence of colloidal platinum or the enzyme catalase. A substance such as platinum in the colloidal state possesses an enormous surface in comparison to its mass, and it is assumed that the forces operative at a surface tend to bring about an increased concentration of material at that surface. As a result of the union between the adsorbed material and the adsorbent, it is possible that strains or stresses are set up in the adsorbed molecules and the latter become more reactive and undergo change with greater rapidity than when in the free state. In other words, the catalyst by adsorption brings about an increased local concentration of the reactant or reactants and at the same time makes these molecules more reactive; i.e., the catalyst activates the substance upon which it acts. Numerous factors are involved in catalytic action, and there is no generally accepted theory of catalysis, but this concept of adsorption and activation does enable us to form a mental picture of the reaction and to explain in simple terms the cause of the increased rate of reaction. A catalyst by itself cannot bring about



a reaction which theoretically cannot occur spontaneously; it can only release the natural inertia to change inherent in the molecule and thus increase the velocity of the reaction. The catalyst appears unchanged after the adsorbed molecule has been decomposed and is ready to unite with more of the material undergoing change; thus a small amount of the catalyst is able to catalyze the change in large amounts of the material unless a product of the reaction is inhibitory to the catalyst, or to the reaction itself, or unless a catalytic poison is added to the reaction mixture. A catalyst cannot alter the equilibrium point of a reaction but merely increases the rate at which that equilibrium is established, generally increasing the rate of reaction in both directions; in other words, catalysis is frequently reversible. A typical example of the reversibility of catalysis is the hydrolysis of butyl acetate by water in the presence of hydrogen ions acting as a catalyst. Acetic acid and butyl alcohol are formed as a result of the reaction, but if one starts with a mixture of acetic acid and butyl alcohol, butyl acetate and water are formed. The rate of hydrolysis or of formation of butyl acetate is very slow or negligible in the absence of the catalytically acting hydrogen ion but is quite rapid in its presence. In either case the same equilibrium is finally established, and we write



Regardless of the amounts of butyl acetate and water or of acetic acid and butyl alcohol initially present, an equilibrium will be established in which the product of the concentration of acetic acid times the concentration of butyl alcohol divided by the product of the concentration of butyl acetate times the concentration of water is equal to a definite numerical value, the equilibrium constant, at a given temperature. The same behavior is noted if the enzyme lipase is employed as the catalyst in place of hydrogen ions, and the same general factors appear to be involved in both ordinary catalysis and enzyme action.

Let us attempt to picture the concept of activation of a molecule by a catalyst or enzyme in the following manner: Suppose that the reacting substance has a certain energy content represented by the level A in Fig 9-1 and that the products of the reaction are at an energy level C. When

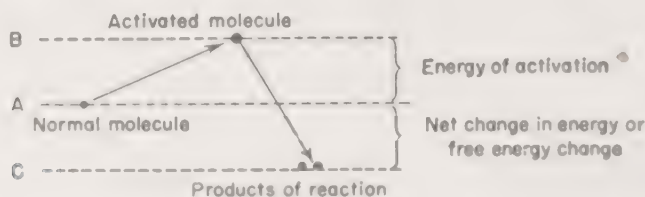


Fig. 9-1. Hypothetical illustration of activation of a molecule.

the reaction occurs there is a free-energy decrease represented by the difference in energy between the levels *A* and *C*. In the presence of a catalyst the rate of the reaction is increased, owing to adsorption and activation of the reactant by the catalyst. As a result of the union between the reacting substance and the catalyst, the energy of the reactant is increased to the level represented by *B*, and the molecule is said to be activated, or rendered more reactive. When the reaction occurs, there is a free-energy decrease from the level *B* to the level *C*, but since the energy of activation is *A* to *B*, this same amount of energy is released when the reaction occurs and hence balances and cancels the original energy of activation. Therefore the actual energy change, even in the presence of the catalyst, is only from *A* to *C*. The total amount of available energy from a given reaction is the same whether the reaction occurs spontaneously or is induced by an enzyme or catalyst.

### ENZYMES

A number of enzymes have been obtained in crystalline form, but their chemical composition is so complex that they are identified and classified on the basis of *what they do* rather than what they are. In general, enzymes appear to consist of two components: (1) a protein portion commonly called a carrier, or *apoenzyme*, involved in, and highly specific for, the adsorption of the foodstuff (substrate) molecule, and (2) a fraction active in carrying out the enzymatically catalyzed reaction and commonly spoken of as a *prosthetic group* or *coenzyme*. The enzymic protein-coenzyme complex is known as a *holoenzyme* (complete enzyme), and the union between the two fractions may be a firm one as in catalase, a protein plus hematin, or it may be a loose one as in the lactic acid dehydrogenase (Fig. 9-2) which consists of a protein plus coenzyme I. Coenzyme I (see Fig. 9-3) is a diphosphopyridinemucleotide (formerly abbreviated as CoI, more commonly now as DPN) and acts as a coenzyme for numerous dehydrogenation reactions, uniting with different apoenzymes. Coenzymes in general may be regarded as shuttle systems, accepting material from one enzymatically activated substrate and transferring it to a different molecule, being regenerated in its original form in the process and ready to continue the cycle. The protein part of the enzyme confers specificity regarding substrate utilized, while the coenzyme is specific for the transport of a product of the enzymatically catalyzed reaction, e.g., hydrogen and electrons, phosphate, acetyl groups, and so on. Coenzymes such as DPN appear to be able to dissociate readily from one protein and shift to another, thus implementing the utilization of the substrate. Metallic ions such as magnesium are also essential to the activity of many enzyme systems, and when this is the

## INTRODUCTION TO THE BACTERIA

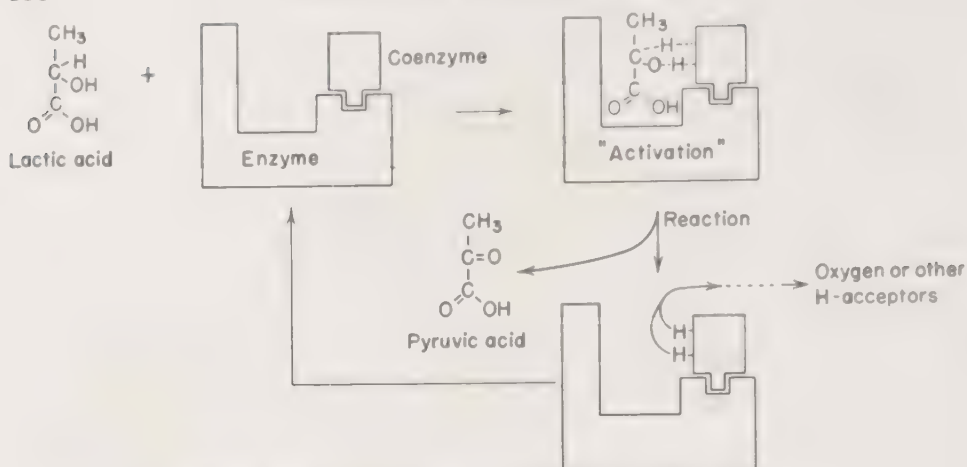


FIG. 9-2. Hypothetical illustration of enzyme action.

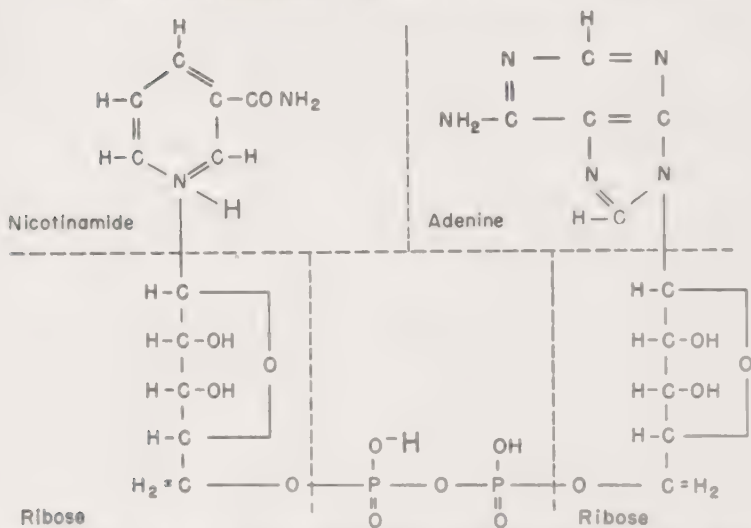


FIG. 9-3. Coenzyme I or DPN, a diphosphopyridine nucleotide. The hydrogen atom in larger, heavier type in the nicotinamide molecule shows where hydrogen is taken up by the coenzyme when it is reduced. Coenzyme II (TPN) differs from coenzyme I by the possession of an additional phosphate group.

case, the ion is known as a *cofactor*. The exact role of the cofactor is not known, but in many cases it appears to function in the binding of the substrate by the enzyme.

The mode of action of an enzyme-coenzyme system may be pictured as illustrated in Fig. 9-2. The enzyme represented possesses two distinct reactive groups spaced relatively close to each other. The substrate molecule, lactic acid in this illustration, is specifically adsorbed by the lactic acid-adsorbing group of the enzyme. The forces holding the molecule together are so altered by this adsorption-activation process that the bonds between two hydrogens and the lactic acid molecule are

so weakened (represented by dotted lines) that these hydrogens are readily given off and taken up by the coenzyme DPN, which is also in specific union with the lactic dehydrogenase. The oxidation product, pyruvic acid, is not firmly bound by the enzyme and is released, thus permitting the adsorption of another lactic acid molecule. DPN, which is reduced as a result of the oxidation of lactic acid, can be oxidized by loss of hydrogen, possibly to another hydrogen acceptor in close spatial relationship to it, or it may dissociate from its union with the enzyme and be attracted to the next hydrogen acceptor in the pathway of oxidation, being replaced by another molecule of the coenzyme in the oxidized form. In either case the reaction could proceed, lactic acid molecules being continuously adsorbed and then oxidized by loss of hydrogen ions (and electrons) to the coenzyme, which in turn is oxidized and ready to oxidize another lactic acid molecule.

Enzymes which are present in the cell and are not secreted into the environment of the cell are spoken of as *intracellular enzymes*, or *endoenzymes*. They may be obtained free from the cell when the latter is disintegrated, and many remain active for a considerable period of time when thus separated. Those which are normally secreted by the cell and whose main site of activity appears to be outside the cell are known as *extracellular enzymes* or *exoenzymes*. Most of the exoenzymes are involved in the hydrolytic splitting of complex substances into simpler units more readily absorbed by the cell. Bacteria capable of liquefying gelatin would be characterized by the secretion of gelatinase, those decomposing cellulose by a cellulase, those hydrolyzing starch by an amylase (see Fig. 9-4), etc.

Enzymes are generally named by adding the suffix *-ase* to the name of the substrate activated by the enzyme or to the type of reaction produced, although the names employed for a number of

enzymes are holdovers from early days and are employed for historical reasons. Examples of various types of enzymes and the reactions they elicit are given in Table 9-1.



FIG. 9-4. Illustration of hydrolysis of starch by bacteria. Starch in the starch-agar plate was not hydrolyzed by *Escherichia coli* (B) and therefore gave a blue color (dark in photograph) with iodine, while no color was produced around the growth of *Bacillus subtilis* (A) which hydrolyzes starch.



TABLE 9-1. A PARTIAL LIST OF ENZYMES AND ENZYMIC ACTIVITY

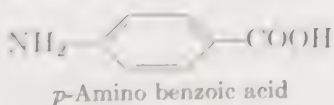
<i>Name</i>	<i>Substrate</i>	<i>Type of reaction</i>
<b>Proteinases</b>	<b>Protein</b>	<b>Hydrolysis</b>
Pepsin	Proteins hydrolyzed to proteoses, peptones, and polypeptides	
Trypsin	Proteins hydrolyzed to proteoses, peptones, and polypeptides	
Gelatinase	Gelatin hydrolyzed to simpler units	
Fibrinolysin	Fibrin decomposed	
<b>Amidases</b>	<b>Amino acids</b>	<b>Hydrolysis</b>
Arginase	Arginine hydrolyzed to urea and ornithine	
Urease	Urea broken down into carbon dioxide and ammonia	
<b>Carbohydrases</b>	<b>Carbohydrates</b>	<b>Hydrolysis</b>
Amylase (Diastase)	Starch hydrolyzed to maltose	
Cellulase	Cellulose hydrolyzed to cellobiose	
Maltase	Maltose hydrolyzed to glucose	
Sucrase (Invertase)	Sucrose hydrolyzed to glucose and fructose	
<b>Esterases</b>	<b>Fats</b>	<b>Hydrolysis</b>
Lipase	Fats hydrolyzed to fatty acids and glycerol	
Phosphatase	Hydrolysis of phospholipids	
<b>Phosphorylases</b>	<b>Carbohydrates and phosphates</b>	<b>Phosphorylation or dephosphorylation</b>
Adenosine phosphorylase	Adenosine triphosphate	Phosphorylation of glucose, etc.
<b>Oxidases</b>	<b>Hydrogen carriers</b>	<b>Oxidation</b>
Indophenol (Cytochrome oxidase)	Cytochrome	Oxidation
<b>Dehydrogenases</b>	<b>Oxidizable foodstuff</b>	<b>Oxidation</b>
Glucose dehydrogenase	Glucose oxidized by loss of hydrogen	
Lactic acid dehydrogenase	Lactic acid oxidized by loss of hydrogen	
<b>Peroxide utilization</b>		
Catalase	Hydrogen peroxide decomposed to oxygen and water	
Peroxidase	Hydrogen peroxide activated as an oxidizing agent	
<b>Decarboxylases</b>	<b><math>\alpha</math>-Keto acids</b>	<b>Carbon dioxide split off</b>
Pyruvic decarboxylase	Pyruvic acid split to carbon dioxide and acetaldehyde	
Amino acid decarboxylase	Amino acids split to carbon dioxide and organic residue	
<b>Transferase</b>		
Transaminase	Amino and keto acids	Transfer of amino group
Transacetylase	Acetate	Transfer of acetyl group

Only a few of the known enzymes have been listed in Table 9-1, and we cannot consider these enzymes in detail here. It is desirable, however, to consider briefly various factors influencing enzyme action and then to consider the activity of enzymes as illustrated by a typical series of reactions common to many organisms, both unicellular and multicellular

**Specificity of Enzyme Action.** The compound which is activated by an enzyme is spoken of as the *substrate* of that enzyme, and in general only one compound is adsorbed and activated by a specific enzyme. Frequently this enzyme-substrate specificity is so complete that only one optical isomer of a particular compound is utilized by the enzyme, a fact observed with entire organisms by Pasteur in his early studies on the optical isomers of tartaric acid. Specificity in other cases is not as restricted as we have just considered, some enzymes being able to catalyze the utilization of compounds having the same chemical grouping or structure in common. For example, certain protein-splitting enzymes, proteases, may hydrolyze various proteins or protein degradation products containing the  $\text{—CO—NH—}$  linkage into their amino acid constituents. On the other hand a particular enzyme may adsorb compounds having the same general structure but may not be able to activate these compounds. This is illustrated by the enzyme succinic acid dehydrogenase, which catalyzes the oxidation of succinic acid to fumaric acid. Malonic acid,  $\text{COOH}\cdot\text{CH}_2\cdot\text{COOH}$ , possesses the same general structure as succinic acid,  $\text{COOH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$ , and is adsorbed by succinic acid dehydrogenase, but it is not activated. It tends to remain at the succinic acid enzyme centers of the cell and by blocking the enzyme inhibits the oxidation of succinic acid. Malonic acid for this reason can be considered as a cellular or enzyme poison.

**Enzyme Inhibitors.** Many substances can inhibit the action of enzymes, a few by competitive inhibition of the malonic acid-succinic acid type, others by a specific inhibition of a chemical group, such as the sulfhydryl ( $\text{—SH}$ ), in the enzyme complex, and still other substances or physical agents can exert a nonspecific inhibition or destruction of enzymes. Competitive inhibition is involved when the degree of inhibition is dependent upon the ratio of the inhibitor to the substrate, not upon the total amount of the poison. Inhibition of the noncompetitive type is generally complete above a certain critical concentration of the inhibitor and is not relieved by additional substrate.

We have given one example of enzyme inhibition of the competitive type, but there is another example that might well be mentioned at this point. The bacteriostatic activity (inhibition of bacterial growth and activity) of the sulfonamide drugs has been explained in terms of competition between the drug and an essential metabolite (an intermediate compound of metabolism essential for the cell) for the enzyme involved in the use of the metabolite. Sulfanilamide apparently inhibits the utilization of *p*-aminobenzoic acid by the bacterial cell, the structural rela-



tionship between these compounds being such that both are adsorbed at the same enzyme center. The sulfa drug is not activated and hence blocks the enzyme to which it is adsorbed. Attempts, some successful, have been made to develop other chemotherapeutic agents on the basis of competitive inhibition, but unfortunately there is so much similarity between the enzymes of bacteria and of mammalian cells in general that the drug frequently competes with the enzymes of the host as well as with those of the parasite.

Many heavy-metal ions, such as those of mercury and silver, are able to inhibit the action of enzymes, in some instances at least, by combination with  $-SH$  groups. This action is frequently reversible; in the case of mercury the latter can be removed by precipitation as the insoluble sulfide, with restoration of enzymic activity. Cyanides, carbon monoxide, and apparently sodium azide inhibit the action of the oxidases by combination with iron in these enzymes. Chloroform and the urethans inhibit the activity of dehydrogenases. A wide variety of agents such as phenol, alcohols, and numerous other compounds, both organic and inorganic, apparently exert a nonspecific inhibitory action which may lead to a denaturation of the enzyme protein. Other factors such as hydrogen-ion concentration and temperature influence the activity of enzymes, and in addition the concentrations of both enzyme and substrate must be taken into account as well as the accumulation of end products of the specifically catalyzed reaction.

**pH.** The hydrogen-ion concentration of the medium has a marked effect on the rate of enzyme action (see Fig. 9-5). There is an optimum pH or pH range for every enzyme, and at values greater or less than the optimum, enzyme activity decreases. When the concentration of hydrogen ions, or of hydroxyl ions, becomes too great, the enzyme is inacti-

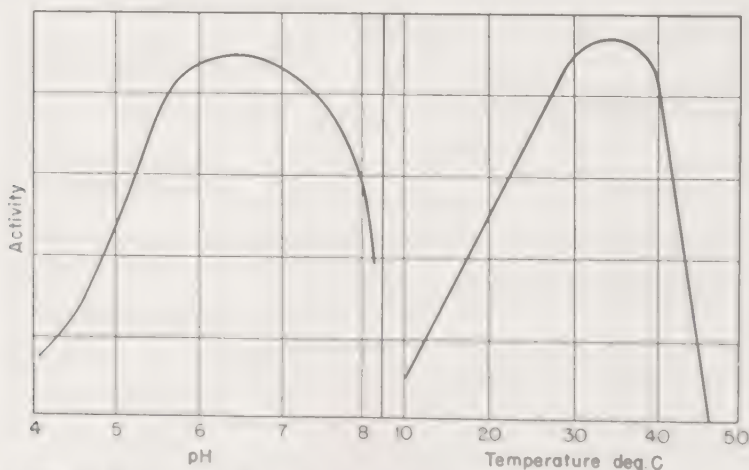


FIG. 9-5 The influence of pH and of temperature on the rate of enzyme action.

vated. The influence of pH on enzymic activity is paralleled by its influence on growth, growth occurring only in that pH range in which the essential enzymes exhibit marked activity.

**Temperature.** Just as there is an optimum pH for the activity of any one enzyme, likewise there is an optimum temperature or temperature range (see Fig. 9-5). In general, enzyme activity increases with increase in temperature up to the optimum temperature, the rate being approximately doubled or trebled for each  $10^{\circ}\text{C}.$  increase in temperature. The rate of enzyme action rapidly decreases as the temperature is increased above the optimum, and at still higher temperatures enzymes are inactivated quite readily. The optimum temperature depends upon the particular enzyme and in some instances upon the source of the enzyme. For example, the enzymes of thermophilic bacteria must have a different temperature optimum than those present in bacteria growing at ordinary temperatures.

**Enzyme and Substrate Concentration.** In general, the rate of enzyme action is proportional to the concentration of the enzyme as long as there is sufficient substrate present to keep the enzyme saturated. This, of course, holds true only if the pH is held constant and if products of the reaction, or of side reactions, are not inhibitory. The production of ethyl alcohol, for example, would be inhibited by the presence or accumulation of too great a quantity of the alcohol in the medium.

Likewise the rate of enzyme action is in many instances proportional to the substrate concentration when the latter is present in low concentrations. As the concentration of the substrate is gradually increased, the reaction velocity increases less rapidly than the concentration until further increase in concentration has little or no effect. Too high a substrate concentration frequently is inhibitory, primarily as a result of osmotic-pressure effects. Some enzymes are saturated at very low substrate concentrations, others at only relatively high concentrations, and the concentration of the enzyme itself must be considered at the same time. Just as enzyme and substrate concentrations influence reaction velocity, so likewise the concentrations of cells and of foodstuffs influence the growth and activity of bacteria.

**Types of Enzymatically Catalyzed Reactions.** To recapitulate, we have considered that enzymes are "active organs" of the cell and that in their activities they are aided by cofactors, often simple ions such as magnesium, and by coenzymes, the protein-coenzyme-cofactor complex making up an "enzyme system." The activity of the enzyme system is markedly influenced by the concentration of hydrogen ions and of substrate, by the temperature, the presence of specific enzyme poisons, and the concentration of products of the reaction. Hence any factor which influences enzyme action influences the activity of the cell as well.



Now suppose yeast cells are growing in a medium containing glucose. We have seen that, when glucose is synthesized by the green plant, the glucose has a molar free-energy content higher by approximately 686,000 cal. than that of the carbon dioxide and water from which the sugar was formed with the aid of sunlight, chlorophyll, and the plant enzymes. Hence, according to the laws of the conservation of energy, as much energy is available when sugar is broken down under the same concentrations and pressures into its components, carbon dioxide and water, as was required for its synthesis. If glucose were oxidized by the yeast cell in the same manner that it undergoes oxidation in a flame, 674,000 cal. of heat energy would be unleashed in a haphazard and probably lethal manner.

It is the function of the enzymes to take the foodstuff molecules, to activate them, and to direct the reactions they undergo so that energy and building material are made available in an organized manner for the cells. The following general types of enzymatically controlled reactions are known to be involved in the utilization of carbohydrates, fats, or proteins by the living cell:

1. *Hydrolysis*: the hydrolytic breakdown of complex molecules to simpler ones utilizable by the cell



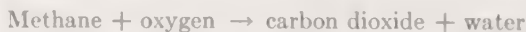
2. *Phosphorolysis*: similar to hydrolysis except that phosphoric acid is substituted for water



3. *Phosphorylation and dephosphorylation*: the addition or removal of phosphate groups to or from a molecule, generally by means of the adenylic acid system, which serves as a means for transfer of energy from one reaction to another



4. *Oxidation and concomitant reduction*: the removal of hydrogen from one molecule and its transfer to another molecule, or the gain and loss of oxygen, respectively



5. *Carboxylation or decarboxylation*: the addition to or removal from a molecule of a molecule of carbon dioxide



6. *Hydration or dehydration*: the addition to or removal from a molecule of a molecule of water



7. *Amination or deamination*: the addition to or removal from a molecule of an amino group



8. *Transfer reactions*, the transfer of an amino group from an amino acid to a keto acid



or the transfer of other groups

9. *Isomerization*, the conversion of one compound into another of the same chemical composition but of different structure



Most of the enzymatically catalyzed types of reaction listed above will be illustrated further as we consider the metabolism of microorganisms in this and in the following chapter. Stress will be placed throughout on the utilization of glucose, but the same general principles apply to the utilization of other sugars, fats, and amino acids. In general, the various foodstuffs are broken down into simpler units, only one or a very limited number of key intermediates of metabolism being produced, from which metabolism proceeds along a common pathway.

## THE UTILIZATION OF FOODSTUFFS

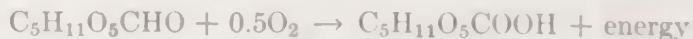
The utilization of foodstuffs by bacteria and other cells involves a considerable number of reactions and enzymes in reaction chains. The energy-providing reactions in these chains act in such a manner that the energy stored in the molecules is either utilized or liberated in small portions without the damage that would result if the molecule were oxidized as in a flame. The general nature of the energy-providing reactions was considered in the preceding chapter, and at this time we will consider the broader aspects of cellular metabolism. Much information is available concerning the dissimilatory reactions elicited by the cell, while less is known concerning the assimilatory ones. The processes of assimilation and dissimilation are intimately connected and can be considered analogous to the orderly series of events involving the provision of energy, of raw materials, of machines, and of directive forces which occur during operation of a modern industrial plant. Each step in the procedure has a definite function, and when one step is inhibited, the entire process is disturbed. We will consider an over-all and very simplified picture of metabolism with particular reference to the universal foodstuff, glucose. Various pathways of metabolism will be discussed in the following chapter with particular reference to the different metabolic groups of bacteria. *Emphasis should be placed throughout on the general nature of the reactions involved and the principles of these reactions; the various chemical formulas and equations being of interest only to those who are chemically-minded.*

The problem confronting us is how the cell is able to break down organic foodstuffs into waste products, at the same time utilizing a por-

tion of the energy and building material bound in the food for cellular purposes. The cell may not be permeable to large molecules, such as starch or gelatin, that it is potentially capable of using as food. These, if they are to be utilized, must be broken down into smaller molecules outside the cell by its extracellular enzymes. Many of the smaller molecules pass into the cell readily, while some do so only with the expenditure of energy. Once the foodstuff has entered the cell, its utilization is controlled by the nature of the cell's enzymes and to some extent by environmental factors. A relatively simple foodstuff such as glucose can be utilized in a variety of ways, even in the same cell. Different pathways of utilization employed by different species of bacteria are particularly evident under anaerobic conditions.

Glucose may undergo condensation reactions in the cell, reacting either with itself or with other sugar molecules to form more complex carbohydrates comprising part of the chemical structure of the cell. Or it may be broken down into simpler units, some of which may enter the synthetic reactions catalyzed by the cell's enzymes. For example, glucose may be oxidatively dissimilated with the formation of pyruvic acid ( $\text{CH}_3\text{COCOOH}$ ), some of which under the influence of an enzyme may take up ammonia with the formation of alanine ( $\text{CH}_3\text{CHNH}_2\text{COOH}$ ), one of the amino acids present in the cell's proteins. Glycerol and fatty acids may be formed from glucose in other reactions and combined to form fats. Other substances are synthesized in similar ways. The energy required for these syntheses comes, for the most part, from transfer reactions involving high-energy phosphate bonds, the transfer of which is mediated by the ATP system. These bonds, as we have seen, are generated during the course of oxidation reactions. There are two major pathways for the oxidation of glucose in the cell. One, the Warburg-Dickens or pentose route, involves preliminary oxidation of the sugar to a sugar acid, which is further dissimilated and oxidized. The other, the Embden-Meyerhof pathway, involves considerable change in the sugar molecule, followed by splitting to form trioses (3-carbon compounds) before any oxidation occurs. Both pathways may be utilized in the same cell. *E. coli*, for example, may utilize up to 40 per cent of the glucose oxidized via the Warburg-Dickens pathway and the remainder over the Embden-Meyerhof one.

In the Warburg-Dickens pathway for dissimilation of glucose, a number of reactions occur during which glucose is oxidized to gluconic acid. This reaction can be represented in a simplified manner as

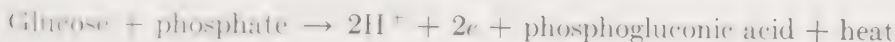


Actually phosphorylation and other reactions are involved, and the end result is that the cell gains in ATP content due to the oxidation of hydro-

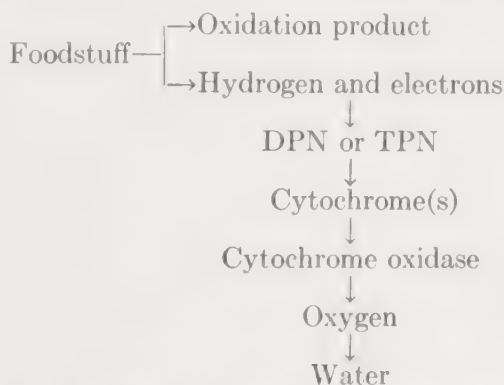


gen from the glucose. Other reactions, including oxidations, decarboxylation with the formation of a 5-carbon or pentose sugar, splitting of the molecules, condensations, and so on, occur until all of the original glucose molecule has been converted into cellular substance or degraded to carbon dioxide and water.

If we analyze the oxidation of glucose to gluconic acid we find that the oxidation can be represented as



glucose acting as a hydrogen and electron donor, its aldehydic group being oxidized to a carboxyl (acid) group. Oxidation involves concomitant reduction, the hydrogen and electrons being transferred in a coupled reaction to a coenzyme of oxidation, generally TPN in this stage of the Warburg-Dickens pathway. This reduced coenzyme in turn is oxidized by loss of hydrogen and electrons to other acceptors in a "bucket brigade," which ultimately brings them into union with oxygen as the "final" hydrogen acceptor under aerobic conditions. The carriers in this chain are flavoproteins and cytochromes, and they, together with the original coenzyme, undergo alternate reduction and oxidation, thus being used over and over again. Without going into the details involved, a typical biological oxidation chain can be represented as



Under anaerobic conditions some compound (or compounds) must substitute for oxygen as the final oxidizing agent. This hydrogen acceptor may be derived from the compound undergoing dissimilation or it may be an entirely different substance, organic or inorganic, present in the medium. Generally the bucket brigade is not involved anaerobically in the transfer of hydrogen and electrons, the reduced coenzyme reacting directly with the oxidizing substance.

We can illustrate both aerobic and anaerobic respiration (fermentation) if we consider the over-all aspects of the Embden-Meyerhof pathway for the dissimilation of glucose. This mechanism operates in many



species in the same basic manner up to the pyruvic acid stage under either aerobic or anaerobic conditions. In essence it consists of a series of enzymatically catalyzed reactions during which the glucose molecule is phosphorylated, undergoes change in structure, and is split into two triose compounds. Oxidation occurs now, with the formation of phosphoglyceric acid, hydrogen and electrons being given up to DPN. Under aerobic conditions these are passed along the bucket brigade to oxygen; under anaerobic conditions the oxidation of the reduced DPN is accomplished by compounds derived from glucose, the particular substance(s) being dependent on the enzymatic make-up of the cells involved. The phosphoglyceric acid produced during the first stages of the Embden-Meyerhof pathway is converted ultimately into pyruvic acid which acts as the final hydrogen acceptor in the lactic acid bacteria (and in muscle under anaerobic conditions), being reduced to lactic acid (see Fig. 9-6).

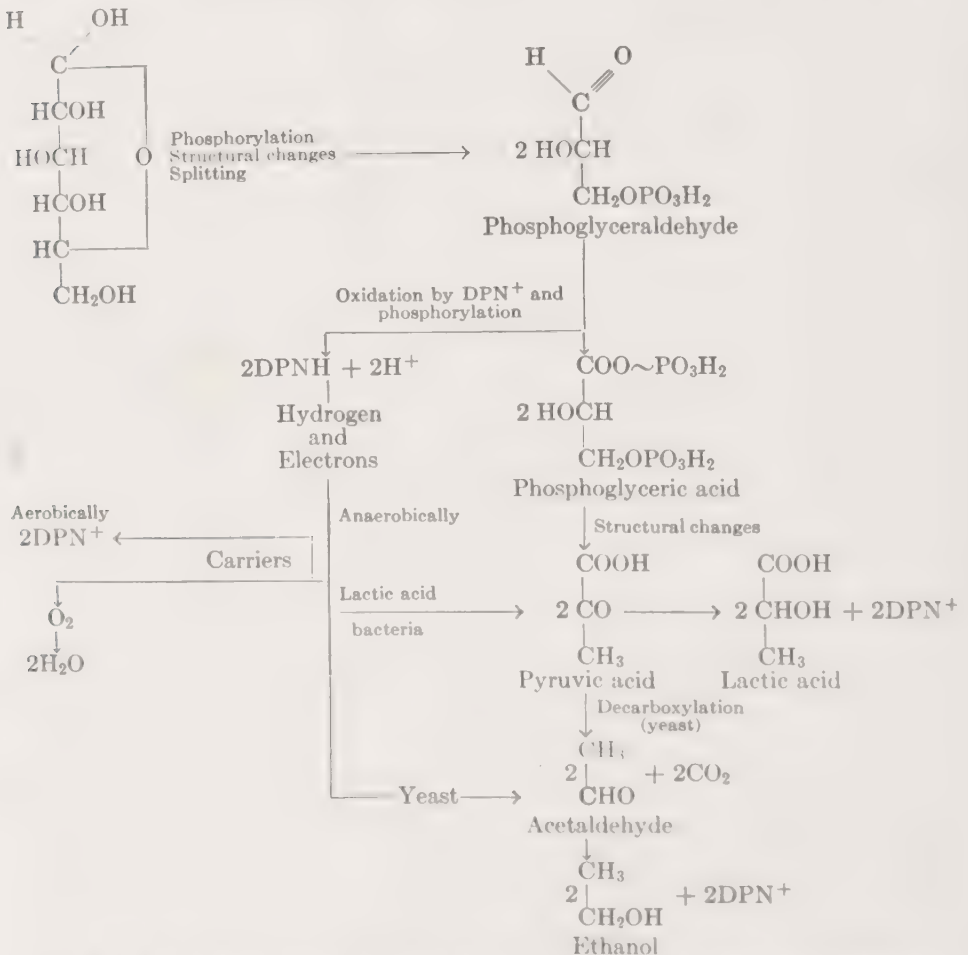


FIG. 9-6. Basic steps of the Embden-Meyerhof pathway for glucose utilization.

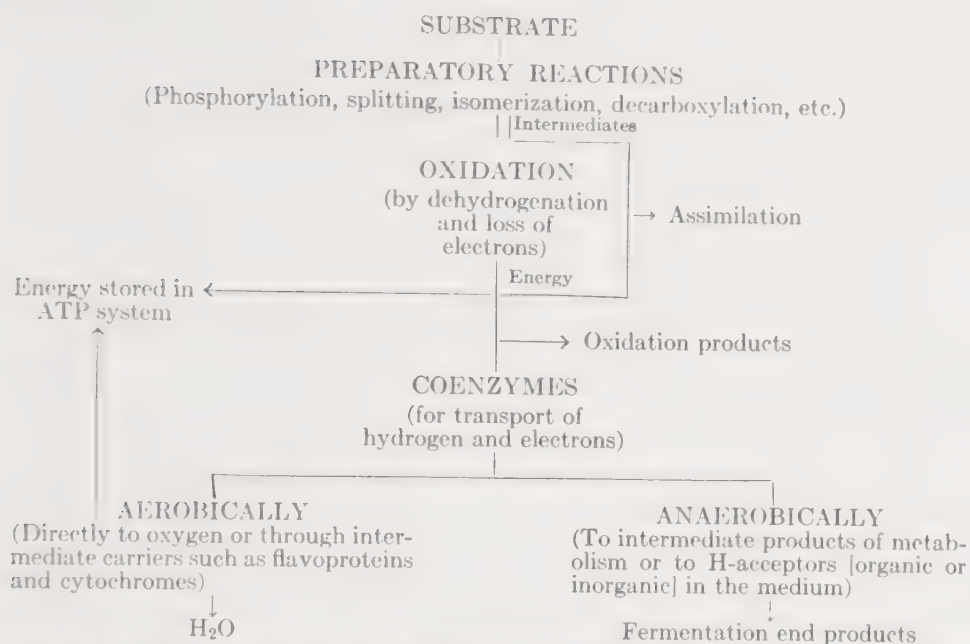


FIG. 9-7. Simplified flow sheet for biological oxidations. During the course of these dissimilatory reactions compounds are formed that can be used in the assimilatory processes.

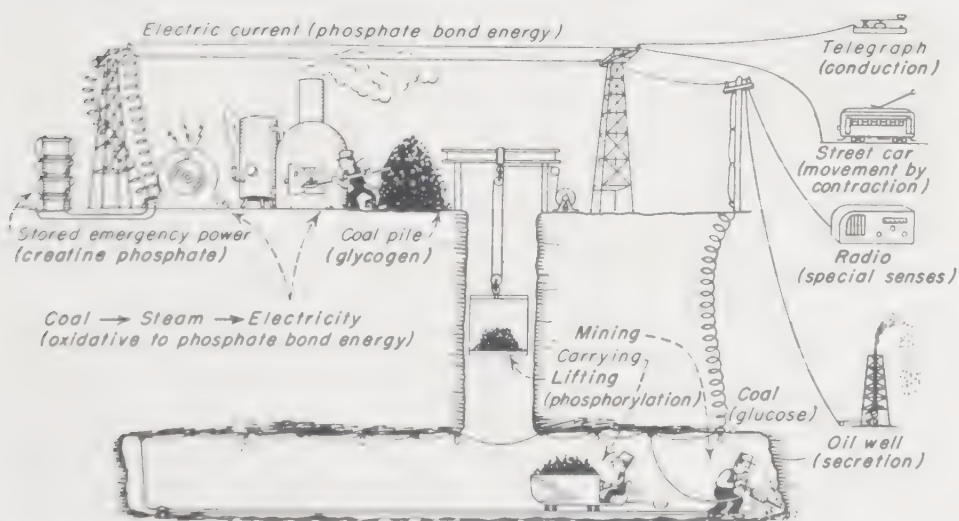
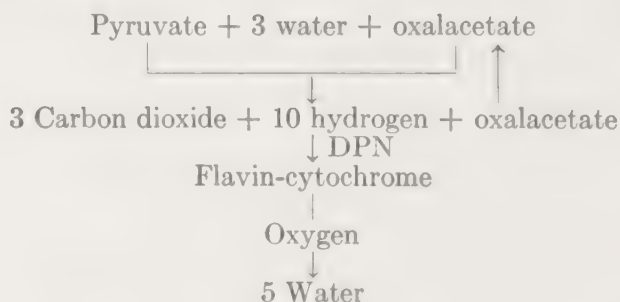


FIG. 9-8. An analogy to the liberation, transfer, and utilization of metabolic energy. (From "Carbohydrate Metabolism," courtesy of S. Soskin and R. Levine and the University of Chicago Press, Chicago.)

In yeast the pyruvic acid is decarboxylated, giving rise to carbon dioxide and acetaldehyde, the latter compound oxidizing the reduced DPN and being reduced with the formation of ethyl alcohol. In many bacteria other substances can be formed from the sugar molecules, some of which act as oxidants, and hence give rise to a variety of fermentation products outlined in the next chapter. It should be remembered that while these chemical events are taking place, high-energy phosphate bonds are formed as a result of the oxidations; also, some of the sugar, or molecules derived therefrom, may be converted into cellular substance. A simplified flow sheet for these reactions is presented in Fig. 9-7 and an analogy to the general picture in Fig. 9-8.

What happens to dissimilation products such as pyruvic acid under aerobic conditions? A portion is assimilated and the remainder is converted into simpler compounds ultimately. In many species pyruvate is oxidatively decarboxylated with the formation of carbon dioxide and an acetyl group in union with coenzyme A. Acetyl-coenzyme A reacts with oxalacetic acid to form citric acid which is converted—in the so-called citric acid, tricarboxylic acid, or *Kreb's cycle*—by decarboxylations, oxidations, and other reactions into carbon dioxide and water, together with a new molecule of oxalacetic acid to continue the cycle. Some synthesis of cellular matter may start from intermediates produced during the course of the cycle. In one revolution of this cycle, three molecules of carbon dioxide are liberated per molecule of pyruvate dissimilated, and ten hydrogen atoms, some from water that is taken up during the course of the cycle, are started on the flavin-cytochrome pathway to oxygen. Or, in summary,



Some of the individual reactions in this cycle are indicated in Fig. 9-9. It is apparent that complex series of events are involved in cellular metabolism, but at the same time these processes are orderly ones and are used in common by widely divergent forms of life.

**Recapitulation of Aerobic and Anaerobic Respiration.** To recapitulate, the principles and mechanisms of biological oxidations are the same under both aerobic and anaerobic conditions. They consist primarily,

After preparatory changes in the foodstuff molecules, of a loosening of the bonds between certain hydrogen atoms and the remainder of the foodstuff molecule under the influence of a specific dehydrogenase. Oxidation is brought about by a transfer of these hydrogen atoms and the electrons involved to a specific hydrogen carrier, generally DPN, which is intimately associated with the dehydrogenase. The coenzyme in turn is oxidized by a flavoprotein enzymic system, which transports the electrons to the cytochrome pathway to oxygen. Under anaerobic conditions these carriers are soon saturated with hydrogen and/or electrons and no longer are able to function. Some compound or compounds other than molecular oxygen must then act as the hydrogen and electron acceptors from the foodstuff undergoing oxidation if cellular respiration is to continue. Since the foodstuff is only partially oxidized under anaerobic conditions, a considerable amount of energy remains bound in the end products of fermentation, and therefore much less energy is available to the cell per mole of foodstuff fermented than is available when an equal amount is oxidized to carbon dioxide and water. The adenylic acid system serves as a means of transfer of energy from exergonic to endergonic reactions.

Other microorganisms possess still other types (or variations) of enzymic systems—phosphorylases, mutases, isomerases, decarboxylases, dehydrogenases, hydrogen transportases, etc.—involved in the dissimilation of their foodstuffs, and as a result we find that different species may employ metabolic pathways other than those just considered, or may employ deviations therefrom. This may lead to the formation of several substances which can act as the final hydrogen acceptors in anaerobic dissimilation with the consequent production of a variety of alcohols, acids, or other organic compounds as the end products of a particular fermentation. In many instances inorganic compounds can be reduced, e.g., nitrates to nitrites, and with a few species gaseous hydrogen itself is an end product. The foodstuffs utilized, and the products of their dissimilation, are dependent on the enzymic structure of the species and serve as the basis of our classification of bacteria according to their biochemical properties.

We have just considered that the pathway of fermentation does vary with the species eliciting the fermentation, but, to complicate matters, it may also vary with the same organism under different environmental conditions. In the normal alcoholic fermentation by yeast, carried out under acidic conditions, a small amount of glycerol is always produced. This can be explained on the basis that 3-phosphoglyceraldehyde, which normally serves as a hydrogen donor, also acts—poorly—as a hydrogen acceptor under acidic conditions, one molecule being oxidized while a second accepts the hydrogen and is reduced to  $\alpha$ -glycerophosphate. The latter compound is then hydrolyzed with the formation of glycerol and



free phosphate. It appears that 3-phosphoglyceraldehyde can be reduced more readily with increasing pH, and in an alkaline environment glucose is fermented by yeast with a conversion of approximately one-half of the sugar to glycerol, the other half to alcohol and carbon dioxide. This shift in the end products of fermentation with change in hydrogen-ion concentration of the culture medium has been employed under wartime conditions for the production of glycerol from fermentable carbohydrates. However, the difficulty of separating the glycerol from the fermentation mixture makes the process economically unfeasible in normal times.

It should also be noted in passing that a given species may undergo change, variants being produced with different fermentation characteristics. Hence any nonlethal change, cellular or environmental, which influences the enzymic structure or activity of the cell may produce an alteration in its fermentative or general metabolic behavior. Fortunately, from the viewpoint of the biochemical classification of bacteria, these variations from the normal pathways generally occur within rather narrow limits.

**Assimilation.** In the preceding discussion on respiration, stress was placed on the dissimilation and oxidation of food material. It might appear that catabolism is the main activity of the cell and that anabolic activities are of secondary importance, being connected in some vague manner with the catabolic ones. Various studies have shown that in experiments of relatively short duration with nonproliferating cells, a considerable portion of the substrate is converted into cellular material; that the amount assimilated depends both upon the nature of the substrate and of the species; and that for a particular combination a relatively definite quantitative relationship exists between the amount of substrate oxidized to carbon dioxide and water and the amount assimilated. Balance sheets indicate an over-all relationship that can be represented, for 50 per cent assimilation of glucose, as



Some organisms, particularly algae, may assimilate 90 per cent or more of the substrate disappearing from the suspension medium, while most bacteria assimilate 25 to 50 per cent of the foodstuff. The extent of assimilation is much lower under anaerobic conditions. The initial product(s) of assimilation approximate(s) the general composition of carbohydrate,  $\text{CH}_2\text{O}$ , regardless of the nature of the substrate. This does not imply, however, that carbohydrate is the only product of assimilation; it simply indicates that cellular material has an over-all composition approximating a ratio of carbon-hydrogen-oxygen of 1-2-1, neglecting the small amounts of nitrogen, phosphorus, sulfur, and so on present in cellular material. Similar relationships are noted in actively proliferat-

ing cultures, although the amount of substrate assimilated appears to be somewhat lower than that noted with cell suspensions. This may be the result of secondary reactions, waste products being created during the conversions of primary products of assimilation into the numerous end products of cellular syntheses.

Growth represents the synthesis of complex compounds, proteins, fats, carbohydrates, nucleic acids, and so on, from, in many instances, a relatively simple organic compound and salts present in the medium. It was believed that synthesis took place to a considerable extent by the reversal of hydrolytic reactions under particular conditions existing in the cells. Such syntheses can occur but probably do so only to a limited extent. Phosphorolytic reactions also occur in the cell, and these are more readily reversible than are the hydrolytic ones. This suggests, and experimental findings lend support to the concept, that phosphorylative synthesis is employed to a considerable extent in cellular metabolism.

Intermediates of different molecular weights and configurations are formed during the oxidation of glucose over the Embden-Meyerhof and the Warburg-Dickens pathways and also in the tricarboxylic acid cycle, and these compounds can serve as starting points for various syntheses (see Fig. 9-9). Some of these compounds are formed in union with ap-

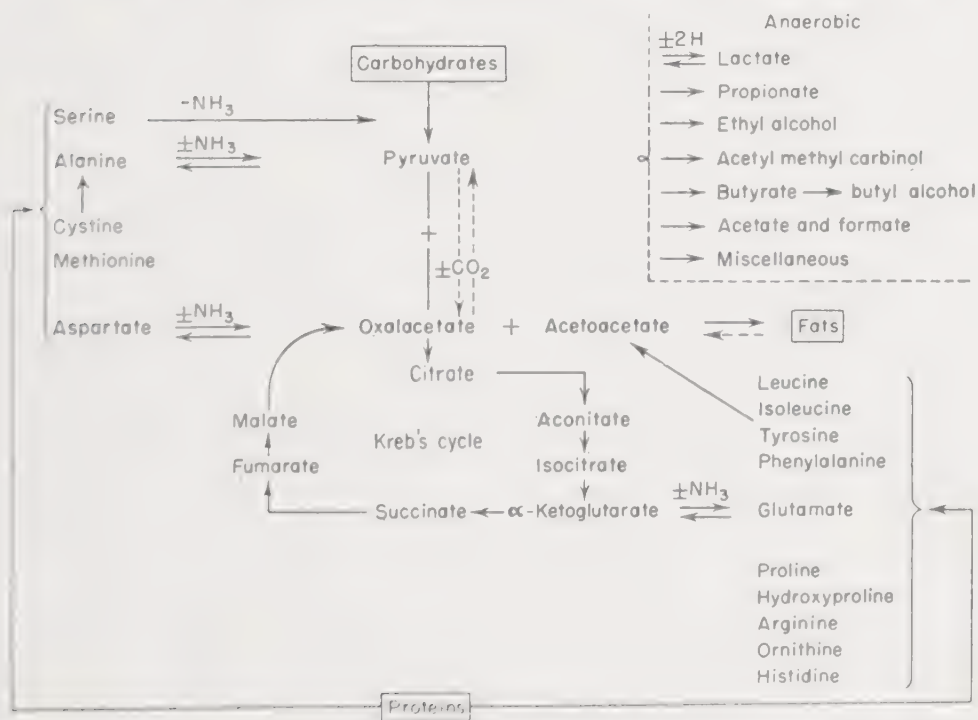


FIG. 9-9. A schematic representation of possible general relationships between assimilation and dissimilation of proteins, fats, and carbohydrates.

propriate coenzymes and are transferred as groups to acceptor molecules. For example, one acetyl-coenzyme A complex can donate or transfer an acetyl group to another acetyl-coenzyme A molecule, with the liberation of one molecule of coenzyme A and the formation of acetoacetyl-coenzyme A. Reduction, further condensations, and so on, lead to the formation of long-chain fatty acids which can be coupled to glycerol to form fats. Protein and polysaccharide syntheses may proceed in similar ways, starting from amino acids or monosaccharides formed in other synthetic reactions.

In summary we can picture metabolism as a highly complex process in which cellular material is formed from the building materials present in the medium, or from substances arising during the course of degradation of the substrate molecules. Both the assimilatory and the dissimilatory reactions are under direct control of the cell's enzymes. Chemical changes—generally oxidations—occurring during the course of the dissimilatory reactions provide the energy required for synthetic and other activities of the cell, energy transfer being mediated primarily by the ADP-ATP system. Excess energy in the form of heat and waste products such as carbon dioxide and water are given off at the same time. The energy present in the chemical bonds in the original foodstuff is liberated in series of reactions which take place in steps and in orderly manner. The reactions are frequently reversible; they may involve systems operative in a cyclic manner; and energy from them is made available in and transferred by the ATP-ADP system. Many of the dissimilatory reactions are well known; those of the assimilatory processes are less well known, but information concerning them is increasing. From what we know of both processes, if the reactions are to occur in an orderly manner it is apparent that metabolism must consist of a wide variety of reactions coordinated in time and space, coordination being accomplished primarily through the agency of the enzymes which catalyze these reactions and impart order and direction to them. This leads to the conclusion that many of the enzymes must be fixed in position within the cell, and, as we shall see later, the ability of a cell to form the particular battery of enzymes characteristic of a given species is intimately associated with and controlled by the genes of that cell.

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## CHAPTER 10

### METABOLIC GROUPS OF BACTERIA

Knowledge of the nutritional requirements and metabolic activities of bacteria is of value in the classification of these organisms and in the development of an understanding of their activities. In Chap. 8 the bacteria were divided into four groups on the basis of their general carbon and nitrogen requirements. In another method of classification, on the basis of their most outstanding modes of nutrition, the bacteria may be divided into two basic groups, *autotrophic* and *heterotrophic*. The term autotrophic is employed here in its broadest sense to indicate that in general these organisms are self-sufficient; they are not dependent on other organisms for the synthesis of their foodstuffs since they can synthesize all their cell substance starting with water, inorganic salts, and carbon dioxide (or bicarbonates) as the constituents of the culture medium. Actually no organism is completely self-sufficient, as in time the supply of an essential substance, e.g., sulfur, in the environment, could be depleted and the organism would then become dependent on another for the conversion of material containing sulfur into sulfur compounds utilizable by the former species. This is well illustrated by the various cycles of the elements, which will be considered in particular in Chap. 15 and which are so important in the economy of nature. The heterotrophs on the other hand require at least one preformed organic substance more reduced than carbon dioxide as their main source of carbon and are therefore dependent upon other forms of life for the provision of essential organic foodstuffs. These two groups can in turn be divided into subgroups on the basis of the most characteristic types of respiration. Again it must be borne in mind that a classification of this sort is not absolute; it only indicates trends. There is no sharp line of demarcation between the various groups. This classification, together with the main characteristics of the organisms in each group and typical examples, can be represented as follows:



# A GENERAL CLASSIFICATION OF BACTERIA ON THE BASIS OF THEIR OUTSTANDING NUTRITIONAL AND RESPIRATORY CHARACTERISTICS

## AUTOTROPHS

### *Chemosynthetic Autotrophs*

Nutrition: Multiply in an inorganic medium in the absence of reduced carbon compounds, carbon being assimilated from carbon dioxide.

Respiration: Aerobic for most species, energy being obtained from the oxidation of inorganic compounds generally highly specific for particular genera.

Respiratory enzymes: Those involved in the oxidation of the inorganic energy source are unknown; other enzymes similar to those in heterotrophic forms.

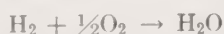
*Examples: Nitrosomonas europaea*



*Thiobacillus thioparus*



*Hydrogenomonas pantotropha*



### *Photosynthetic Autotrophs*

Nutrition: Most species can multiply in an inorganic medium, while a few require growth factors and/or reducible organic matter, carbon being assimilated primarily from carbon dioxide.

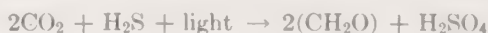
Respiration: Anaerobic, energy for the reduction of carbon dioxide to cellular material being obtained from light.

Respiratory enzymes: Enzyme systems involved in the photochemical reduction of carbon dioxide, other enzymes similar to those in heterotrophic forms.

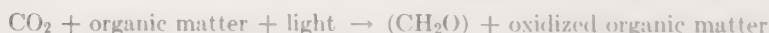
*Examples: Green sulfur bacteria, Chlorobium limicola*



Purple sulfur bacteria, *Thiopedia rosea*



Nonsulfur purple and brown bacteria, *Rhodospseudomonas palustris*



## HETEROTROPHS

### *Aerobes*

Nutrition: Multiply only in the presence of suitable organic matter, nutritional requirements may be simple or complex, two genera able to utilize nitrogen gas.

Respiration: Aerobic oxidation of organic matter, generally to carbon dioxide and water.

Respiratory enzymes: Similar to those of other plants and animals. Complete cytochrome system.

\* The formula  $(\text{CH}_2\text{O})$  represents the empirical composition of the product of photosynthesis.

Examples: *Mycobacterium tuberculosis*,† *Bacillus anthracis*†

*Mycobacterium phlei*, *Bacillus subtilis*, *Acetobacter aceti*

Nitrogen fixers: *Azotobacter chroococcum*, *Rhizobium leguminosarum*

### Facultative Anaerobes

Nutrition: Multiply only in the presence of suitable organic matter; nutritional requirements tend to be more complex than those of the strict aerobes.

Respiration: Aerobic oxidation of organic matter, generally to carbon dioxide and water or incomplete oxidation (fermentation) of carbohydrates or amino acids.

Respiratory enzymes: May be as complete as those of the strict aerobes. Certain components of cytochrome system may be absent. Catalase may or may not be present.

Examples: *Salmonella paratyphi*,† *Diplococcus pneumoniae*,† *Erwinia amylovora*,‡  
*Escherichia coli*

### Anaerobes

Nutrition: Multiply only in the presence of suitable organic matter; nutritional requirements generally more complex and more specific than those of the aerobes.

Respiration: Anaerobic oxidation of organic matter with marked accumulation of waste products of carbohydrate or amino acid fermentation.

Respiratory enzymes: Cytochrome system, catalase, and peroxidase absent. Dehydrogenases more limited in number than in the aerobes.

Examples: *Clostridium tetani*,† *Clostridium sporogenes*

Most species of heterotrophic bacteria can lead an anaerobic existence, and it is therefore simpler to consider the heterotrophs as strict aerobes and as fermentative organisms on the basis of their most characteristic types of respiration. The two groups and the main types of fermentation can then be represented as:

### CHEMOHETEROTROPHS

1. Bacteria with obligatory oxidative catabolism—strict aerobes ..... *Acetobacter aceti*
2. Bacteria with fermentative powers
  - a. Production of "mixed" acids ..... *Escherichia coli*
  - b. Production of 2,3-butylene glycol ..... *Aerobacter aerogenes*
  - c. Alcoholic fermentation ..... *Clostridium botulinum*
  - d. Butyric acid fermentation, including the production of butyl alcohol and acetone ..... *Clostridium butylicum*
  - e. Propionic acid fermentation ..... *Propionibacterium shermanii*
  - f. Lactic acid fermentation ..... *Lactobacillus lactis*
  - g. Proteolytic fermentation (amino acid degradation) ..... *Clostridium lentoputrescens*
  - h. Miscellaneous reductions ( $\text{CO}_2$ ,  $\text{NO}_3$ ,  $\text{SO}_4$ ) ..... *Micrococcus denitrificans*

Consideration of these main physiological groups will lead to a better understanding of the metabolic activities of bacteria.

† Animal pathogens.

‡ Plant pathogens.

**Chemosynthetic Autotrophs.** We have considered autotrophic bacteria in the strict sense of the term as those organisms which synthesize all their essential metabolites from inorganic sources, using energy from the oxidation of inorganic matter in the case of the chemosynthetic forms, from light in the photosynthetic bacteria, for the reduction of carbon dioxide. Carbon dioxide is the source of carbon, and in the case of the chemosynthetic forms only one inorganic substance or a closely related group of substances will serve as the fuel for the cell. However, to any broad statement made about the bacteria, there are generally one or more exceptions. Certain species of the hydrogen-oxidizing bacteria require a trace of thiamin for growth in an otherwise inorganic medium, while the photoheterotrophic bacteria, which are primarily autotrophs, utilize organic matter as an ultimate source of hydrogen for the photosynthetic reduction of carbon dioxide to cellular material. Also a limited number of species commonly considered as autotrophs can grow in ordinary culture media and oxidize the organic matter as a source of energy.

In the discussion to follow, let us consider the autotrophic bacteria as being those forms characterized by the ability to synthesize the bulk, probably more than 99 per cent, of their cellular carbon from carbon dioxide, remembering that certain of the bacteria so classified can also grow as heterotrophs. Many or all heterotrophs may be able to assimilate carbon dioxide, but it is not their chief source of carbon. Nature in general does not have sharp lines of demarcation between groups of organisms or between various phenomena exhibited by the groups, while man in his thinking tries to establish definite boundaries as an aid in his study of natural phenomena. We must not allow more or less arbitrary attempts at strict classification and definition to interfere with the development of a broad understanding of the bacteria and other forms of life about which we still know very little.

**Discovery of the Autotrophs.** As early as 1862 Pasteur suggested that the oxidation of ammonia to nitrates in the soil was the result of microbial action. In 1887 Schloesing and Muntz demonstrated the probable biological production of nitrates from ammonia in sewage flowing through a long tube filled with sand and chalk. For a number of days the effluent contained ammonia and no nitrates; then ammonia disappeared from the effluent and was replaced by nitrates. The oxidation of ammonia to nitrates (nitrification) was favored by aeration and by a slightly alkaline environment and stopped when a germicidal agent such as chlorine vapor was passed through the tube. Nitrates again became evident in the effluent several days after garden soil was added to the tube. Nitrification stopped on heating the tube to 100° C. and was resumed within a few days after adding soil washings to the sewage flowing through the tube. These studies of Schloesing and Muntz strongly im-

ulated that microorganisms were responsible for the oxidation of ammonia to nitrates, since nitrification was blocked by chemical or physical agents inimical to life. They also observed that nitrites were present at times in the effluent and suggested that the oxidation took place in two steps, ammonia to nitrites and nitrites to nitrates. They were unable to demonstrate the oxidation of ammonia in pure cultures of bacteria, yeasts, or molds.

In 1875 Cohn demonstrated that the refractile granules (Fig. 10-1) observed in *Beggiatoa*, a genus of soil and water bacteria, consisted of elementary sulfur, and he assumed (1) that the sulfur deposited in these bacteria was a partial oxidation product of hydrogen sulfide oxidation, and (2) that under certain conditions the reaction could be reversed, this intracellular sulfur being reduced to hydrogen sulfide by *Beggiatoa*. In 1887 Winogradsky quite conclusively demonstrated that the sulfur granules arose from the oxidation of hydrogen sulfide by *Beggiatoa* and that these organisms did not elicit the reverse reaction, the reduction of sulfur to sulfide. Furthermore in the absence of hydrogen sulfide the intracellular sulfur was oxidized to sulfate. Hydrogen sulfide formation occurred only when other species of bacteria were present. Sulfur reappeared in the cells of *Beggiatoa* when hydrogen sulfide was supplied to the culture. Winogradsky concluded that the oxidation of sulfide to sulfur and of sulfur to sulfate supplied these bacteria with the energy required for growth as the oxidation of organic matter serves as the energy source for the more common bacteria. Shortly thereafter he concluded that the deposition of ferric hydroxide by the iron bacteria in water containing ferrous salts was due to a comparable oxidation process.

Verification of the concept of the synthesis of cellular matter from carbon dioxide with energy derived from the oxidation of inorganic matter came around 1890 in Winogradsky's studies on the nitrifying bacteria. He observed that organic matter was at times inhibitory to the growth of the sulfur bacteria, and he therefore attempted to cultivate the nitrate producers in as simple a medium as possible, using tartrates as the carbon source. Little or no oxidation of ammonia was observed when this solution was inoculated with rich garden soil. When tartrates were omitted from the medium, nitrification was observed. On repeated transfer in the inorganic medium (potassium phosphate and carbonate, magnesium sulfate, ammonium chloride, and the impurities present in these salts),

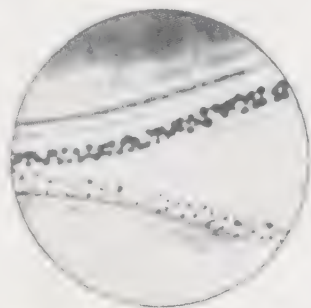


FIG. 10-1. *Beggiatoa alba*. (From Nonak, "Documenta Microbiologica," Gustav Fischer, Jena, 1927.)



he obtained a culture believed to be pure. No growth occurred when material was streaked from these cultures onto gelatin. He next tried streaking the organisms on his inorganic medium solidified by the addition of silicic acid instead of gelatin or agar, and on this silica-gel medium he succeeded in isolating colonies of nitrifying bacteria. He was finally able to show that the oxidation took place in two steps, ammonia to nitrites and nitrites to nitrates, each step being the result of the catabolic activity of a different organism. Two genera, *Nitrosomonas* and *Nitrosococcus*, capable of oxidizing ammonia to nitrite and one genus, *Nitrobacter*, capable only of oxidizing nitrite to nitrate were recognized.

**General Nature of the Autotrophs.** Winogradsky's observations were soon confirmed by various workers, and the existence became established of autotrophic forms capable of growth in the dark in mineral media and deriving energy from the oxidation of sulfide, sulfur, thiosulfate, or analogous selenium compounds, or from the oxidation of ammonia.

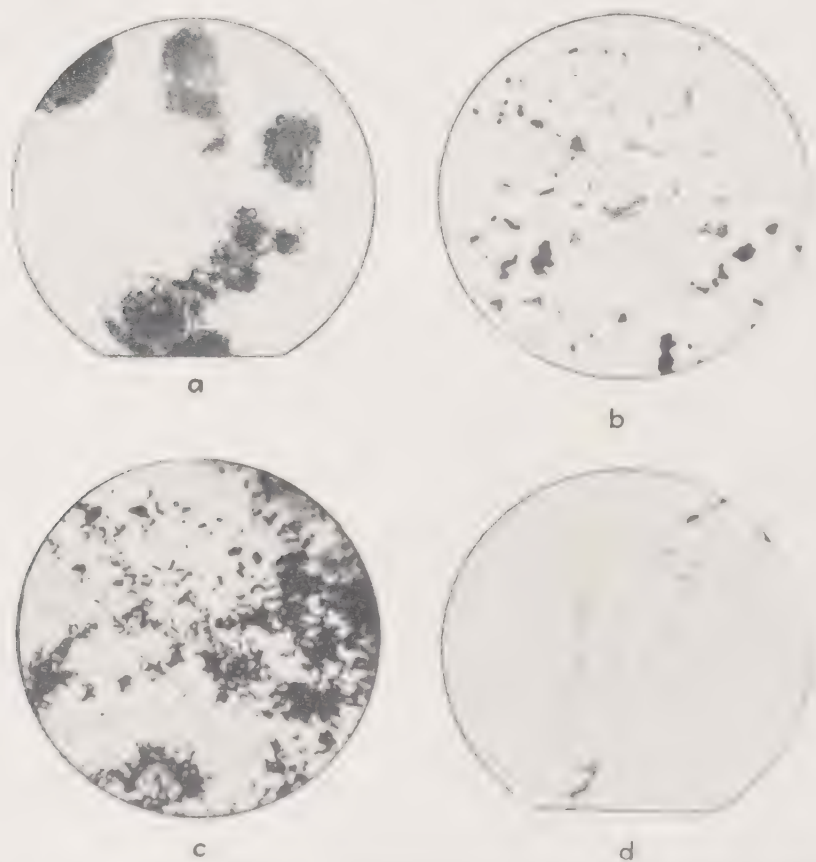


FIG. 10-2. (a) and (b) Species of ammonia-oxidizing bacteria (*Nitrosomonas*) and (c) and (d) nitrite oxidizers (*Nitrobacter*). (From Nowak, "Documenta Microbiologica," Gustav Fischer, Jena, 1927.)

nitrite, hydrogen, and possibly ferrous or manganous salts. The nutrition of these forms is analogous to that of the green plants with the exception that energy is derived from oxidation reactions rather than from light. Winogradsky attached much importance to the concept that organic matter was inimical to the growth of the autotrophic bacteria. More recent studies indicate that a low concentration of certain organic compounds may promote growth of many of the species of autotrophic bacteria and that facultative chemoautotrophs do exist. The essential point is that there are a number of bacteria capable of growth in the dark in a strictly inorganic medium with carbon dioxide as the main or only source of carbon, energy being obtained from the oxidation of specific types of inorganic matter.

An autotroph of particular interest to biologists is *Thiobacillus thiooxidans*, which utilizes the oxidation of sulfur to sulfuric acid as a source of energy, or



Actually, attempts have been made to employ this organism for the production of sulfuric acid, but they have met with little or no success. This bacterium will not grow in a neutral solution; its pH optimum for growth is between 2 and 3.5, and it will grow in solutions having a pH below 1.0 with a sulfuric acid content greater than 5 per cent. Such a solution would be toxic to most forms of life. Another point of interest with this and other sulfur bacteria is the mechanism of sulfur transport into the cell. Sulfur is insoluble in water, but it appears to be dissolved in fat or oil droplets at the surface of the cell and to enter the cell in solution in this oil.

Other sulfur bacteria multiply in less acidic or in slightly alkaline solutions, and one species, *Thiobacillus denitrificans*, is able to develop anaerobically, sulfur or sulfide being oxidized at the expense of nitrates, which are reduced to nitrogen. Selenium compounds may be oxidized by some or all of the sulfur bacteria. The sulfur bacteria are of particular importance in the soil, where they convert sulfide and sulfur into sulfate, which can be assimilated by the green plants and converted by the plants into the sulfur-containing organic compounds essential in both plant and mammalian nutrition.

Autotrophic forms of the genus *Hydrogenomonas* can obtain energy for growth from the oxidation of gaseous hydrogen, but most species of the hydrogen-oxidizing bacteria will also grow as heterotrophs. Certain iron-oxidizing bacteria have been considered to be autotrophs, oxidizing ferrous carbonate, for example, to ferric hydroxide, or



Doubt exists as to the actual autotrophic nature of these bacteria, but ferric hydroxide is deposited in sheaths surrounding these organisms, as mentioned in Chap. 7. The reason for this deposition of iron remains unknown. The iron-depositing bacteria are for the most part rather complex morphological forms or are associated with complex structures in comparison with the autotrophs and are very difficult to cultivate and to study in the laboratory. They may have been responsible for the deposition of considerable quantities of bog iron, e.g., in the Great Lakes area. At the present time they frequently cause considerable trouble in water distribution systems in which, by their deposition of iron-containing material, they tend to clog the pipes, to discolor the water, and on decomposition to produce bad odors or tastes in the water.

One misconception concerning the autotrophic bacteria is that they do not metabolize organic matter, their internal metabolism (as is also true for photosynthetic organisms) other than the primary reduction of carbon dioxide being overlooked. Recent studies indicate that the autotrophic bacteria contain enzyme systems, such as adenylic acid, cytochromes, and various dehydrogenases, involved in the utilization of organic matter in the heterotrophs. Carbon dioxide assimilation supplies organic matter *within the cells*, and the organic compounds so synthesized within the cell are then used in the various metabolic activities of the organism. It appears that these cells are not permeable to extraneous organic food-stuffs or for other reasons are unable to assimilate them. The utilization of organic matter formed within the cell also occurs in the photosynthetic bacteria and in the green plants. High-energy phosphate bonds appear to be the main mechanism for the transport of energy from the catabolic to the anabolic processes in the autotrophs as well as in the heterotrophs. These considerations indicate the remarkable unity of biochemical activity in nature.

**Photosynthetic Bacteria.** Engelmann, in 1883, observed a marked phototactic response of certain reddish or purple pigmented bacteria, these organisms migrating to definite regions in the spectrum in a distribution similar to that exhibited by motile green algae. From these and other observations he concluded that the purple bacteria were able to carry on a photosynthetic type of existence, although his proof of oxygen production during photosynthetic activity of these forms was far from convincing. The main evidence consisted of the observations that good growth took place only in the light under anaerobic conditions and that a chemotactic influence appeared to attract aerobic bacteria to clumps of the photosynthetic forms, i.e., to a region where oxygen was assumed to be present.

Other photosynthetic forms were observed in ensuing years, but no satisfactory explanation of the photosynthetic behavior of these bacteria

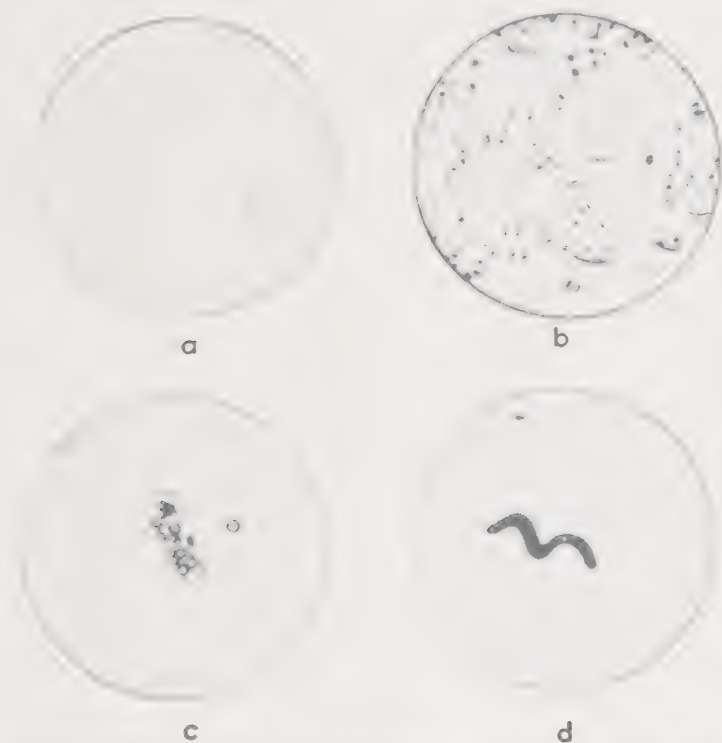


FIG. 10.3 Photosynthetic bacteria: (a) and (b), *Chromatium vinosum*, showing sulfur granules; (c), *C. okenii*; and (d), a thiospirillum. (From Nowak, "Documenta Microbiologica," Gustav Fischer, Jena, 1927.)

was advanced until 1930 when van Niel demonstrated that hydrogen sulfide rather than water appeared to supply the hydrogen needed for the photochemical reduction of carbon dioxide by the Thiobacteriaceae. Sulfur, therefore, rather than oxygen, was an end product of photosynthesis of the photosynthetic sulfur bacteria. Sulfur in turn could serve as a reducing agent in the presence of light and the purple bacteria, being oxidized to sulfate with concomitant reduction of carbon dioxide to cellular material. These reactions were pictured as follows:



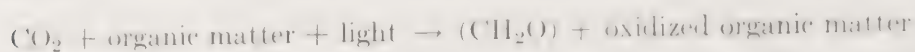
or



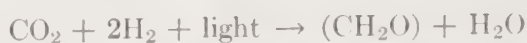
The formula  $(\text{CH}_2\text{O})$  simply represents the empirical composition of the product of photosynthesis. Since multiplication to any extent occurred only in the light and since only one mole of sulfate was produced for every two moles of carbon dioxide assimilated, van Niel concluded that this was a photosynthetic assimilation of carbon, an equivalent amount of carbon assimilation in the case of the chemosynthetic sulfur auto-



trophs requiring the oxidation of forty or more moles of sulfur to sulfate to supply the necessary energy. He demonstrated a similar type of photosynthetic activity in the green sulfur bacteria, with the exception that sulfide was oxidized only to sulfur, which was deposited extracellularly. Muller, in 1933, demonstrated an analogous type of photosynthetic reaction in the nonsulfur purple and brown heterotrophic bacteria, the organic matter serving as a source of hydrogen for the photochemical reduction of carbon dioxide to cellular material, or



In these bacteria, while they do require the presence of organic matter for growth, the carbon assimilated comes from carbon dioxide itself, and actually these organisms are primarily autotrophic in their existence. These forms, the *Athiorhodaceae*, are also capable of growth in the absence of organic matter and in the presence of molecular hydrogen, according to the equation



This is further evidence of the essentially autotrophic nature of this group of bacteria. Actually (see Chap. 8) carbon dioxide is reduced by hydrogen from the photolytic splitting of water, the photosynthetic bacteria reducing the hydroxyl radicals with hydrogen obtained from sulfur compounds, from organic matter, or from gaseous hydrogen, depending on the species involved.

The photosynthetic pigments of the photosynthetic bacteria are related to the chlorophyll of the green plants, but there are essential differences between the bacterial chlorophylls and those of the green plants. These differences are partially reflected in the fact that reducing agents other than water are employed in photosynthesis and that oxygen is not an end product of the reaction. As far as is known, the enzyme systems of these organisms are similar to those of other bacteria and of the green plants.

**Luminous Bacteria.** There are a number of bacteria characterized by the fact that their growth on fish or on decaying organic matter is accompanied by the liberation of light. This is frequently the cause of phosphorescence observed in swamps or in schools of fish in the ocean. This phenomenon of luminescence is not related to photosynthesis but is of interest in that light is emitted during the respiration of these organisms (under aerobic conditions) rather than being absorbed as in photosynthesis. The luminous bacteria emit part of their waste energy as heat, another part as light, which is produced in oxidations through the luciferin-luciferase enzyme system rather than through the cytochromes. Certain chemical reactions not involving enzyme systems also liberate a portion of the energy of reaction as light rather than as heat energy.

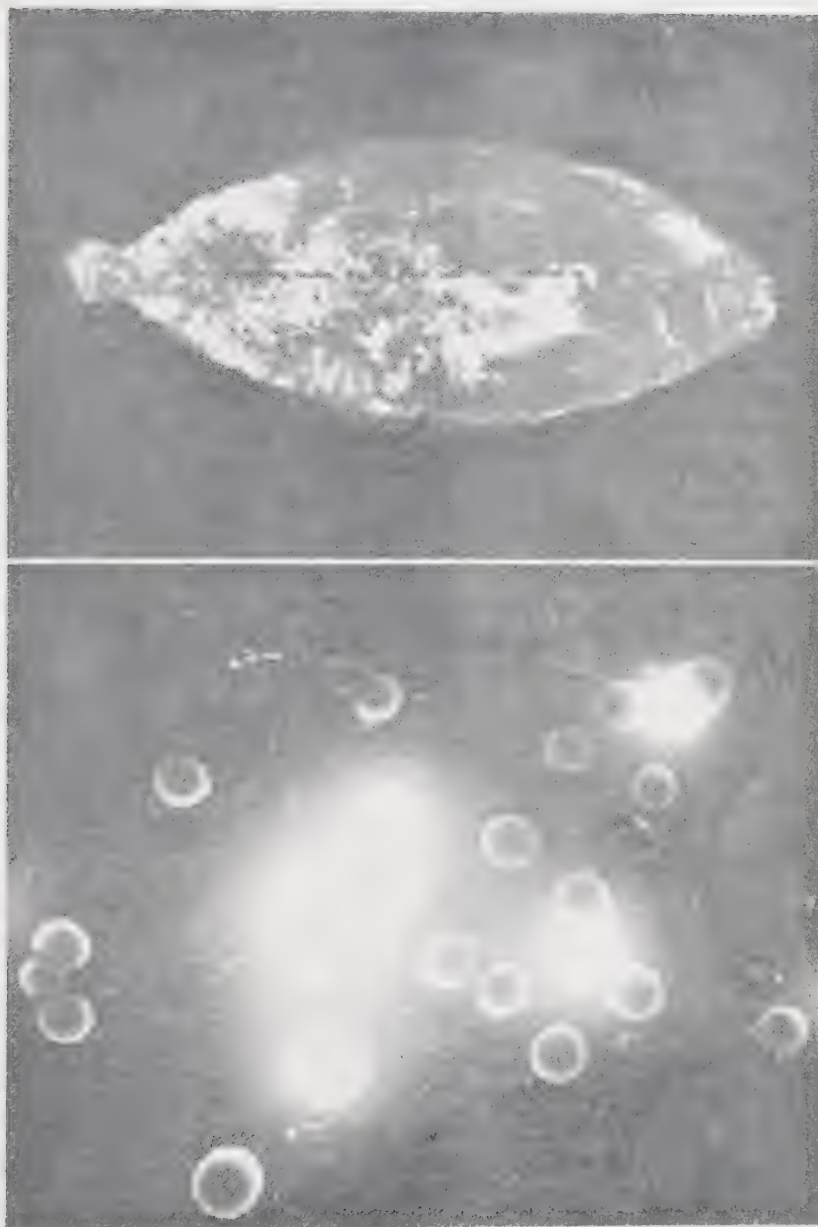
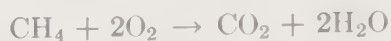
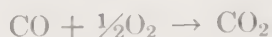


FIG. 10-4. Luminous bacteria on a fish and colonies of luminous bacteria photographed by their own light. (Courtesy of A. Giese.)

**Chemosynthetic Heterotrophic Aerobes.** The autotrophic bacteria capable of multiplying not only in an inorganic medium but also under heterotrophic conditions constitute a group intermediate between the strict autotrophs and the obligate heterotrophs. At least two species of bacteria are known which will grow primarily as autotrophs, but they do require at least one simple compound of carbon for the provision of

energy by oxidation if growth is to occur. One, comparable to the hydrogen-oxidizing bacteria, oxidizes carbon monoxide, the other oxidizes methane:



and utilizing the energy so derived, they apparently convert carbon dioxide to cellular matter in a manner analogous to the autotrophs. The carbon monoxide-oxidizing organism is an oddity amongst the bacteria since it is able to use as fuel a substance toxic to most forms of life. The methane-oxidizing bacterium, *Methanomonas methanica*, is found in the soil, where it probably oxidizes methane formed in anaerobic fermentation in the lower layers of the soil.

The acetic acid bacteria are quite typical of the aerobes with the exception that they do not oxidize all of their substrate to completion. *Acetobacter aceti* is representative of this group of bacteria and is commonly associated with vinegar production, oxidizing a portion of the ethyl alcohol in wines or cider only as far as acetic acid, which accumulates in the medium. *Acetobacter xylinum* is of interest in that the capsule produced by this organism contains a considerable amount of cellulose identical with cotton cellulose in X-ray pattern. (The molds in general also appear to carry out a partial oxidation of a portion of their foodstuffs, as is evidenced by the accumulation of a variety of organic acids and other compounds in the medium which originally contained only one organic substrate such as glucose.) The free-living, nitrogen-fixing bacteria, the genus *Azotobacter*, are obligate aerobes and



FIG. 10-5. *Bacillus brevis*.

have been reported to exhibit the highest rate of metabolism of any organism.  $Q_{10}$ 's (caloric millimoles of oxygen consumed per milligram dry weight of cells per hour) as high as 3,000 having been reported as compared with values of 50 to 300 for most species of bacteria and of 2 to 10 for various mammalian cells. A number of the true bacilli are primarily aerobes, a typical example being *Bacillus brevis*, which is employed for the production of the antibiotics gramicidin and tyrothricin.

**Chemosynthetic Heterotrophic Anaerobes.** The majority of bacteria grow most readily under aerobic conditions, but many species are also

able to multiply either under low oxygen tensions or in the complete absence of gaseous oxygen. These facultative anaerobes, as a result of their ability to multiply under widely diverse conditions of oxygen supply, are found widely dispersed in nature, growing most luxuriantly under aerobic conditions but also multiplying when oxygen is deficient. The strict anaerobes are more limited than the facultative anaerobes, both in numbers and in their ability to adapt themselves to different conditions. These two groups will be considered together, however, since the types of fermentations (see page 207) carried out can be common to both groups. Also the type of fermentation elicited by a facultative anaerobe is more characteristic of the species than its behavior under aerobic conditions. While the production of acetic acid by the acetic acid bacteria (*Acetobacter*) is spoken of as acetic acid fermentation, it is a fermentation only in the broadest sense of the term, in that the oxidation of the foodstuff is incomplete and organic wastes accumulate in the medium. Oxygen, however, is consumed during the production of the acetic acid. The fermentations to be considered here are those which take place without concomitant oxygen consumption.

The products of bacterial fermentations are numerous and varied and depend on the nature of the organism, the pH at which the fermentation is carried out, and upon the nature of the foodstuff—carbohydrate, protein, or fat. The more common products of fermentation can be listed as follows:

Hydrogen,  $H_2$

Carbon dioxide,  $CO_2$

Methane,  $CH_4$

Formic acid,  $HCOOH$

Acetic acid,  $CH_3 \cdot COOH$

Propionic acid,  $CH_3 \cdot CH_2 \cdot COOH$

Butyric acid,  $CH_3 \cdot (CH_2)_2 \cdot COOH$

Pyruvic acid,  $CH_3 \cdot CO \cdot COOH$

Lactic acid,  $CH_3 \cdot CHOH \cdot COOH$

Succinic acid,  $CH_2 \cdot COOH$

$CH_2COOH$

Ethyl alcohol,  $CH_3 \cdot CH_2OH$

Propyl alcohol,  $CH_3 \cdot CH_2 \cdot CH_2 \cdot OH$

Isopropyl alcohol,  $CH_3$

$CHOH$

$CH_3$

Acetone,  $CH_3$

$CO$

$CH_3$

Butyl alcohol,  $CH_3 \cdot (CH_2) \cdot CH_2OH$

Acetylmethylcarbinol,  $CH_3 \cdot CO \cdot CHOH \cdot CH_3$

Amines and other amino acid residues, e.g.,

$CH_3NH_2$  (methylamine)

Needless to say that not all of these substances are formed during a fermentation carried out by a particular species, but the actual number



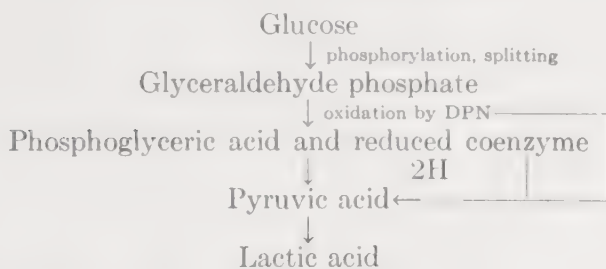
may range from one to eight or ten end products, most or all of which may arise from pyruvic acid formed during the course of dissimilation of the foodstuff.

**Alcoholic Fermentation.** The production of ethyl alcohol and carbon dioxide as a result of the fermentative ability, i.e., the enzymes, possessed by baker's or brewer's yeast was considered in the preceding chapter. A number of bacteria produce small amounts of ethyl alcohol during the fermentation of glucose or other sugars, at times by a pathway apparently different from that in yeast. A smaller number of bacteria, including *Clostridium botulinum*, *Pseudomonas lindneri*, and *Sarcina ventriculi*, ferment carbohydrates with the production of relatively large amounts of ethyl alcohol and carbon dioxide together with smaller amounts of organic acids. *C. botulinum*, for example, ferments glucose with the production of more than one mole each of alcohol and carbon dioxide, approximately one-third of a mole of lactic acid, and two-thirds of a mole of a mixture of acetic and butyric acids per mole of glucose utilized. The pathway of this fermentation has not been elucidated. *Pseudomonas lindneri* carries out a fermentation that appears to resemble alcoholic fermentation by yeast. This species is employed in the preparation of pulque, a fermented beverage derived from the juice of the *Agave* plant.

**Lactic Acid Fermentation.** Many bacteria, including the streptococci, the pneumococci, and the lactic acid bacteria, produce considerable quantities of lactic acid from glucose or other sugars. The lactic acid-producing bacteria are generally divided into two groups, the homofermentative and the heterofermentative. The former group, represented by the lactic acid bacteria of milk such as *Streptococcus lactis* and *Lactobacillus bulgaricus*, produce lactic acid only during the fermentation of sugars. The heterofermentative lactic acid bacteria, on the other hand, form appreciable amounts of carbon dioxide, ethyl alcohol, acetic acid, and glycerol together with large quantities of lactic acid. Lactic acid exists in two optical forms (mirror images), and some lactic acid bacteria produce the dextrorotatory, others the levorotatory isomer, and still other species produce a mixture of the two isomers. This is another example of the specificities exhibited by the bacteria through their enzyme systems. Lactic acid bacteria are employed for the industrial production of lactic acid, over five thousand tons being produced annually in the United States.

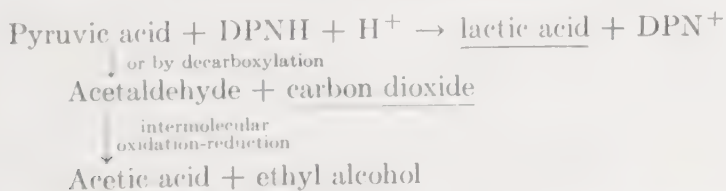
The initial stages of lactic acid production by homofermentative bacteria appear to be identical with those of alcoholic fermentation up to pyruvic acid. These homofermentative bacteria do not possess the enzyme carboxylase and hence are unable to split carbon dioxide from the molecule of pyruvic acid as is done by yeast. Instead the pyruvic acid is reduced to lactic acid. The true lactic acid fermentation can be summarized as follows:

### HOMOFERMENTATIVE LACTIC ACID FERMENTATION (END PRODUCT UNDERScoreD)



The heterofermentative lactic acid bacteria possess additional enzymes, not present in the homofermentative species, capable of utilizing pyruvate in fermentations other than its reduction to lactic acid. Also they may dissimilate glucose via the pentose route. Some species possess a carboxylase by means of which some of the pyruvic acid is decarboxylated to give acetaldehyde and carbon dioxide. Other enzymes are involved in the utilization of the acetaldehyde, one-half of the acetaldehyde molecules being oxidized to acetic acid, the other half reduced to ethyl alcohol. Acetic acid and alcohol, therefore, accumulate in the medium since they cannot be further decomposed under anaerobic conditions and are typical end products, along with carbon dioxide and lactic acid, of the heterofermentative fermentation. This can be represented as follows:

### HETEROFERMENTATIVE LACTIC ACID FERMENTATION OF GLUCOSE (END PRODUCTS UNDERSCORED)

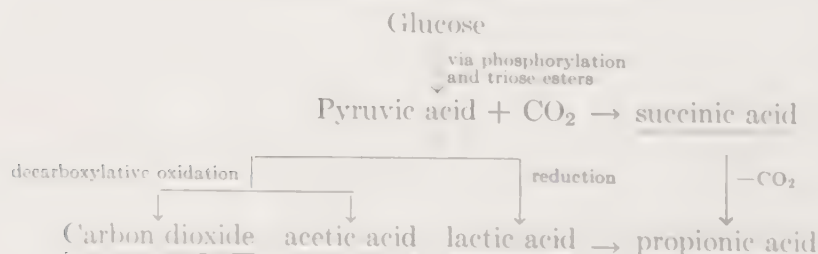


Small amounts of glycerol are generally formed during heterofermentative fermentation, the glycerol arising from the reduction of dihydroxyacetone phosphate as also occurs in alcoholic fermentation. It should be emphasized here that these various reaction schemes are presented not as material to be memorized by the general student but simply to indicate that the various products of the different bacterial fermentations arise as a result of deviations from the main line of fermentation. These deviations are due to differences in the enzymatic pattern or content of the different bacterial species. Why different species vary so in their enzyme content is not readily explainable on the basis of evolutionary development since, for example, there is no apparent advantage in carrying out the heterofermentative as contrasted with a homofermentative type of

lactic acid fermentation. On the other hand, man can be thankful for the heterofermentative types of fermentation since they frequently result in the production of substances adding a more delightful odor or taste to milk products such as butter and cheese than would be imparted by a straight lactic acid type of fermentation. Other fermentations to be discussed in the following pages further illustrate the enzymic complexity of bacteria and the variation in pattern between different species, variations which are taken into account both in applied bacteriology and in the classification of bacteria. It is important to know not only that different types of fermentation occur but also that they reflect different pathways of anaerobic respiration in the different species, pathways which are illustrated by highly simplified schemes of fermentation presented to *illustrate* the discussion.

**Propionic Acid Fermentation.** The *Propionibacter*, or propionic acid bacteria, are commonly found in hard cheeses of the Swiss and Emmentaler types, to which they impart characteristic flavors and the "eyes" or gas pockets in these cheeses. They characteristically ferment sugars with the production of both propionic and acetic acids and carbon dioxide, together with smaller amounts of succinic acid. An analogous type of fermentation is carried out by *Corynebacterium diphtheriae*. A completely satisfactory explanation of the fermentation has not been worked out, but it appears that the various end products arise from pyruvic acid formed as in other fermentations. It is possible that two moles of pyruvic acid may react with each other, one mole being reduced to lactic acid while the other mole is decarboxylated with the formation of carbon dioxide and acetaldehyde, the latter acting as the reducing agent for the formation of lactic acid and as a result being oxidized to acetic acid. Lactic acid in turn could be reduced to propionic acid. These bacteria are also able to fix carbon dioxide with pyruvic acid, thus giving rise to a 4-carbon compound which can be converted to succinic acid. Actually, most or all of the propionic acid may arise from the decarboxylation of succinic acid, evidence for which has been obtained with the aid of heavy carbon or of radioactive carbon as a tracer. The following scheme illustrates possible pathways for the propionic acid fermentation:

PROPIONIC ACID TYPE OF GLUCOSE FERMENTATION  
(END PRODUCTS UNDERScoreD)



**Butyric Acid Fermentation.** This type of fermentation is of considerable practical interest since it was employed during the First World War as a source of acetone and in more recent years in the industrial production of butyl alcohol, over 80,000 tons being produced annually in the United States. Formerly corn meal was employed as the substrate for the fermentation, the butyric acid clostridia employed for the fermentation possessing enzymes capable of hydrolyzing starch to sugar. New strains have been isolated which utilize the sugar in crude molasses more readily than corn meal, and they are being employed to a considerable extent at the present time. This fermentation is even more complex than the ones previously considered since a greater variety of end products is produced. Strains of *Clostridium acetobutylicum* give rise to the production of various amounts of acetic and butyric acids, ethyl and butyl alcohols, acetone, hydrogen, and carbon dioxide. The closely related species (or possibly strain), *C. butylicum*, forms the same end products with the exception that most of the acetone is reduced with the formation of isopropyl alcohol. Environmental factors markedly influence the course of this type of fermentation, this being particularly well illustrated in studies by Davies and Stephenson (see Fig. 10-6) on the influence of hydrogen-ion concentration of the medium and of the accumulation of acetic acid in particular on the course of the fermentation.

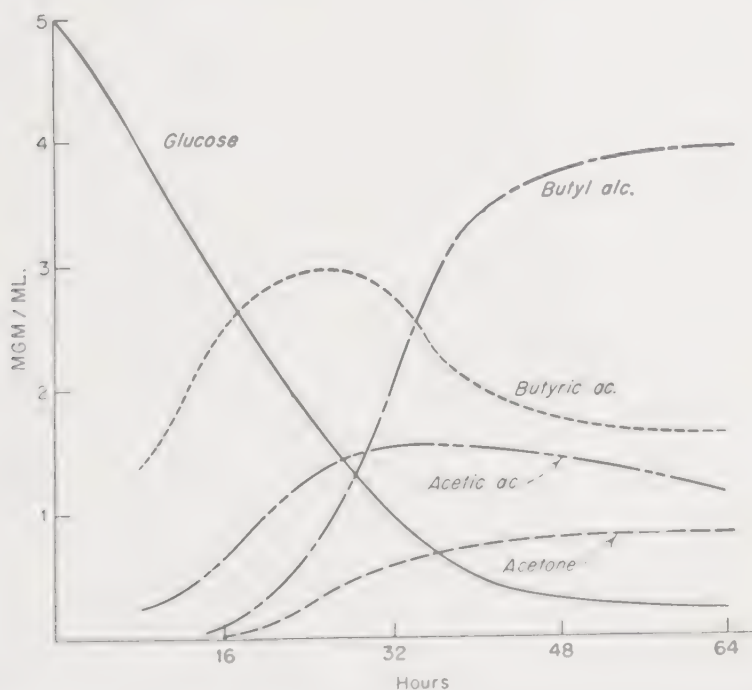
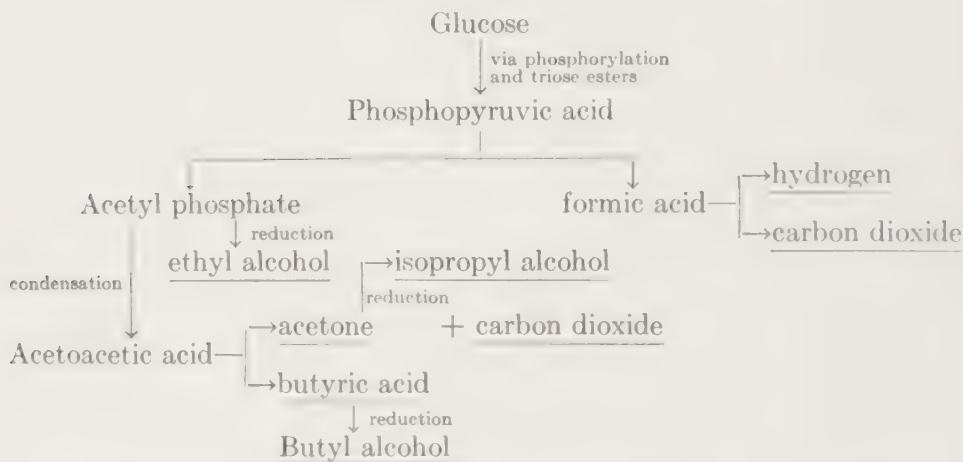


FIG. 10-6. Changes in pH and in end products of fermentation by *Clostridium acetobutylicum* in a glucose medium. [From Davies and Stephenson, *Biochemical Journal*, **35**, 1323 (1941).]



It is apparent that acetic and butyric acids accumulate in the medium during the early stages of growth and that they decrease in amount with time concurrently with the accumulation of butyl alcohol and acetone, the former being produced by reduction of butyric acid, the latter from acetic acid. The studies of the above and other workers suggest that the reduction of the acid to the alcohol is favored at a pH near 4.5. On the other hand, studies with radioactive carbon as a tracer indicate that acetone and butyl alcohol may both arise from a condensation of acetic acid to give acetoacetic acid, which can be decarboxylated to yield acetone or be reduced to butyl alcohol, possibly via butyric acid. There is some evidence to indicate that glucose is phosphorylated and fermented, as in yeast, to the pyruvic acid stage and that pyruvate phosphate is then split with the formation of acetyl phosphate, carbon dioxide, and hydrogen. The various end products could arise from acetyl phosphate as suggested in the following tentative scheme:

**BUTYRIC ACID-BUTYL ALCOHOL-ACETONE TYPE OF  
GLUCOSE FERMENTATION  
(END PRODUCTS UNDERSCORED)**



**Mixed Acids and 2,3-Butylene Glycol Fermentations.** The fermentation of glucose by the coli-aerogenes-typhoid, or enteric, group of bacteria has been worked out in considerable detail, since this group contains many of the more common bacteria, both saprophytic and pathogenic species. The coli type of fermentation is characterized by the production of considerable quantities of organic acids together with hydrogen and carbon dioxide, the aerogenes type by the production of smaller quantities of organic acids and the formation of acetylmethylcarbinol and its reduction product 2,3-butyleneglycol, and the typhoid-dysentery type by fermentations similar to the above types with the exception that gas is not produced during the fermentation, the appropriate enzymes

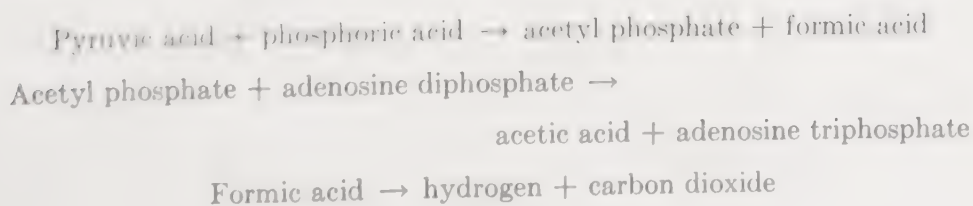
being absent in these species. The end products of the fermentation of glucose by representative species of the enteric bacteria and the relative amounts of each product are presented in Table 10-1.

TABLE 10-1. PERCENTAGE OF CARBON IN END PRODUCTS OF THE FERMENTATION OF GLUCOSE \*

Products	Organism			
	Coli	Aerogenes	Typhoid	Dysentery
2,3-Butylene glycol.....	....	32.4		
Succinic acid.....	20.6	4.4	6.6	7.4
Lactic acid.....	42.4	5.0	60.8	68.4
Acetic acid.....	14.3	3.3	8.6	7.6
Ethyl alcohol.....	14.3	17.7	8.5	7.7
Formic acid.....	0.4	3.2	6.6	5.1
Carbon dioxide.....	7.3	24.0		
Hydrogen (molar basis) ..	= CO <sub>2</sub>	= $\frac{1}{3}$ CO <sub>2</sub>		

\* From doctoral dissertation by M. A. Scheffer, Delft, 1928.

*Coli-Salmonella Type of Fermentation.* The preliminary stages of this type of fermentation appear to follow the pathway of alcoholic fermentation by yeast up to the formation of the triose phosphates. Pyruvic acid carboxylase is absent from members of the coli-typhoid-dysentery group of bacteria, and therefore pyruvic acid will not be decarboxylated with the formation of acetaldehyde. Instead pyruvic acid is split with the formation of acetic and formic acids, the latter being decomposed by the gas-producing enteric bacteria into hydrogen and carbon dioxide under the influence of the enzyme formic hydrogenlyase. Recent studies indicate that pyruvic acid is phosphorolytically split with the formation of acetyl phosphate and formate, the acetyl phosphate being converted into acetic acid by loss of phosphate to the adenylic acid system, with the formation of a high-energy phosphate bond which can be utilized in energy-requiring reactions. The over-all reactions involved in the utilization of the pyruvic acid formed in the normal manner from glucose can be represented as



*Escherichia coli* (see Table 10-1) converts approximately 50 per cent of the fermented sugar into lactic acid by way of pyruvic acid. This suggests that pyruvic acid by itself should be fermented with the formation of lactic acid, but instead pyruvate is fermented as illustrated in the above scheme. This well illustrates both the complexity and the coordination of chemical events in the living cell. Hydrogen is necessary for the reduction of pyruvic acid to lactic acid, and this hydrogen comes from the reduced coenzyme (DPNH) formed in the oxidation of phosphoglyceraldehyde to phosphoglyceric acid during the course of glucose fermentation. When this source of hydrogen is not available, respiration follows a different pathway of pyruvate utilization. Since the reduced coenzyme is also utilized in other reactions catalyzed by *E. coli*, only a portion of the pyruvate formed from glucose terminates as lactic acid, the remainder being utilized as indicated in the preceding equations.

In the previous chapter it was pointed out that yeasts produce some glycerol during the course of alcoholic fermentation, the glycerol arising from the reduction of dihydroxyacetone in a shunt reaction analogous to the above alternate pathways of respiration. Dihydroxyacetone phosphate is also formed during the course of the *coli* fermentation, is reduced to  $\alpha$ -glycerophosphate, and the latter is split with the formation of ethyl alcohol and formic acid rather than being converted into glycerol. Note that in this fermentation, ethyl alcohol arises from a source other than that in the yeast fermentation. Pyruvic acid and dihydroxyacetone compete for hydrogen from DPNH. The relative amounts of ethyl alcohol and of lactic acid produced in the fermentation mixture depend, therefore, to some extent upon the pH at which the fermentation is carried out, since the pH influences the rates of reduction of pyruvate and of dihydroxyacetone phosphate. This again illustrates the influence of environmental factors on microbial activities. These examples are cited not as important facts to be memorized but as examples of the adaptability of bacteria to changes in their environment.

For many years it was believed that succinic acid might arise from a 4-carbon compound formed as a result of the splitting of a portion of the glucose fermented into a 4- and a 2-carbon compound rather than entirely into 3-carbon compounds. Elsdon in 1937 showed that succinate can be formed from pyruvate and that the amounts formed increased as the carbon dioxide concentration in the environment was increased. Shortly thereafter Wood and Werkman demonstrated an actual uptake of carbon dioxide during the fermentation of pyruvate by *E. coli*. This was done with the aid of the heavy isotope of carbon (molecular weight of 13) incorporated as a tracer in carbon dioxide. They demonstrated that the heavy carbon appeared in a carboxyl group of succinic acid according to the equation



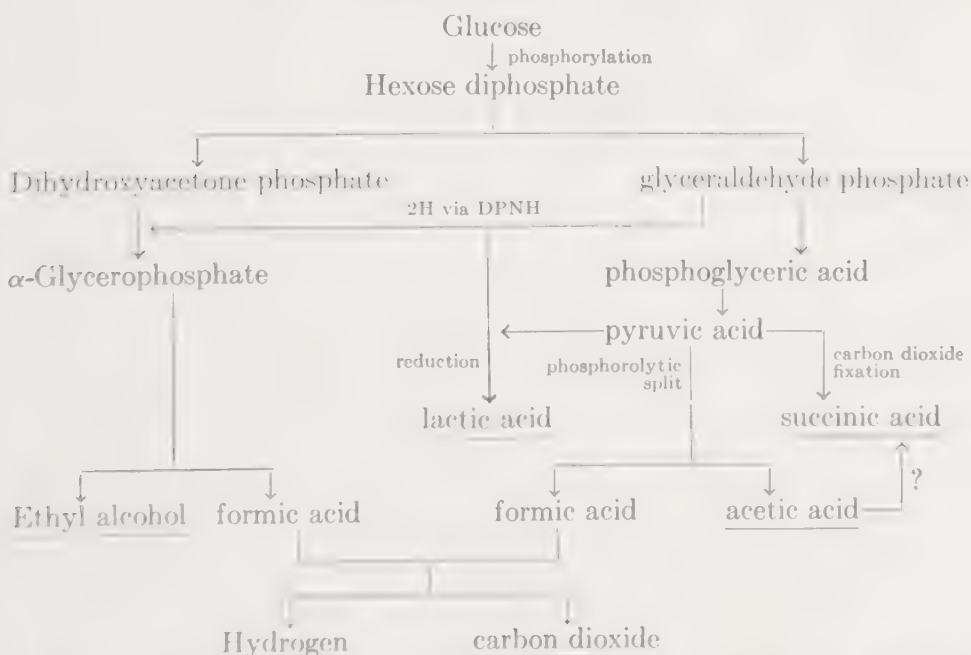
in which C\* represents heavy carbon. They have also demonstrated that succinic acid can be formed as the result of a dehydrogenation and condensation of acetic acid according to the equation



These studies indicate that a four-two carbon split of the glucose molecule as earlier postulated is not essential for the formation of succinic acid.

The various intermediate reactions in the fermentation have not been considered in this discussion, and it is known that in most cases a number of reactions and enzyme systems are involved; e.g., succinate could be formed from pyruvate and carbon dioxide via oxalacetic, malic, and fumaric acids. Stepwise utilization gives the cells greater control of the reactions involved. For our purpose only a generalized scheme of the fermentation reactions is necessary, and the formation of the various end products of glucose fermentation by *E. coli* and many of the salmonellae can be depicted as follows:

COLI-SALMONELLA TYPE OF GLUCOSE FERMENTATION  
(MAIN END PRODUCTS UNDERScoreD)



This scheme indicates that hydrogen and carbon dioxide are produced in equal amounts. The experimentally determined ratio frequently



differs from unity since some of the carbon dioxide may be utilized in the formation of succinic acid. Also some of the hydrogen gas may be utilized, with the aid of the enzyme hydrogenase, in the reduction of intermediates formed during the fermentation of glucose. A ratio of approximately 1.0 is characteristic of the coli fermentation, while a ratio of approximately two parts of carbon dioxide to one of hydrogen is characteristic of the aerogenes fermentation, in which there are two sources of carbon dioxide. This difference is one of the tests employed in differentiating between *Escherichia coli* and *Aerobacter aerogenes*. The above scheme of fermentation also indicates that many of the end products of the coli-salmonella fermentation are acidic in character. Actually sufficient acids accumulate in cultures of *E. coli* to develop the red, acid color (pH of 4.5 or less) of methyl red, the pH indicator incorporated in or added to glucose broth cultures for the test. As we shall see, less acid is produced in cultures of *A. aerogenes*, and therefore the methyl red test is negative; i.e., not enough acid is produced to develop the red color with this organism. Fermentations carried out by many of the salmonellae are similar to the coli fermentation, a differential characteristic being that lactose is generally not fermented by species of the genus *Salmonella*.

*Typhoid-Dysentery Fermentation.* Typical results for the fermentation of glucose by typhoid and dysentery bacteria as presented in Table 10-1 indicate that the end products are similar to those of the coli fermentation with the exception that more lactic acid is produced and that formic acid is not converted into hydrogen and carbon dioxide. Formic hydrogenlyase is not formed by these bacteria. The absence of gas formation serves to differentiate these bacteria from the coli-aerogenes-salmonella fermentation group. The typhoid bacterium, *Salmonella typhosa*, because of these biochemical differences, was formerly placed in a separate genus, *Eberthella*. Other characteristics (primarily antigenic, see Chap. 23) of this organism are so closely related to those of *Salmonella* that it was placed in this genus in the sixth edition of "Bergey's Manual of Determinative Bacteriology." The intermediate stages of fermentation are probably common to all the enteric bacteria.

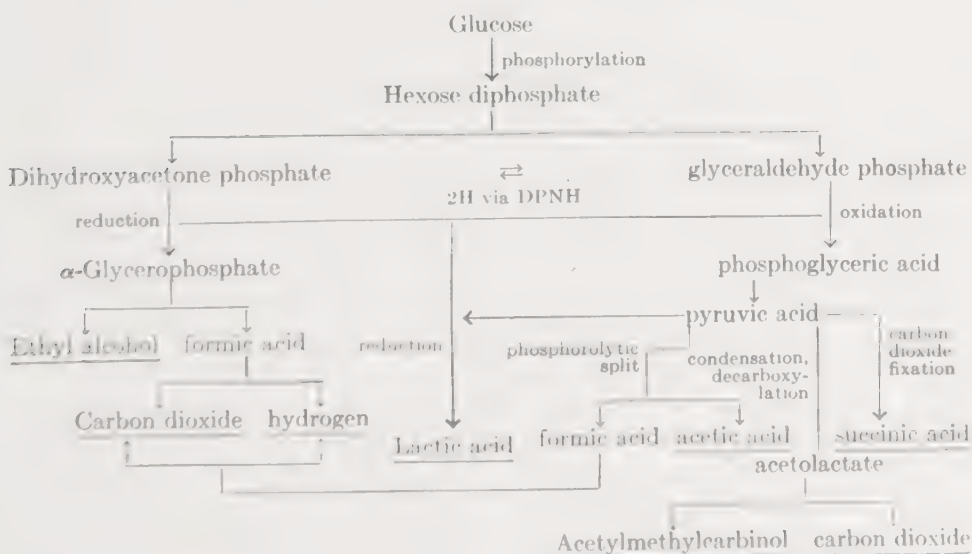
*Aerogenes Fermentation.* The aerogenes type of fermentation is similar to the coli type with the exception of greater production of carbon dioxide, lesser acid formation, and the production of acetylmethylcarbinol (acetoin) or its reduction product 2,3-butyleneglycol, in glucose-broth cultures of *A. aerogenes*. The formation of acetylmethylcarbinol is detected in the laboratory by means of the Voges-Proskauer (VP) test or some modification thereof. In this test, diacetyl,  $\text{CH}_3\cdot\text{CO}\cdot\text{CO}\cdot\text{CH}_3$ , is produced under strongly alkaline conditions by atmospheric oxidation of

acetylmethylcarbinol. The diacetyl then reacts with creatinine to give a pink color, a positive VP test.

Glucose is fermented by *A. aerogenes* and related species primarily via pyruvic acid, which in turn can be further dissimilated by either a phosphorolytic split to give acetyl phosphate and formic acid or by decarboxylation and condensation of two molecules to form acetylmethylcarbinol. The latter reaction occurs at an appreciable rate only after the medium has become somewhat acidic owing both to the former reaction and to the production of lactic acid by pyruvate reduction. Since a considerable portion of the glucose fermented is converted into acetylmethylcarbinol, it is apparent that less acid will accumulate than in cultures of *E. coli*, and hence the methyl red (MR) test is negative. It should be emphasized that a negative MR test does not indicate that acid is not produced, it indicates only that sufficient acid to develop the red color of the indicator is not produced. When the culture is allowed to stand for several days, acetylmethylcarbinol is reduced under anaerobic conditions to yield 2,3-butylene glycol, while it is oxidized to diacetyl if aerobic conditions develop. These substances, and in particular diacetyl, are responsible to a considerable extent for the characteristic aroma of butter.

Small amounts of succinic acid are generally produced in the aerogenes type of fermentation and also considerable quantities of ethyl alcohol, the latter apparently arising from  $\alpha$ -glycerophosphate. These substances are probably formed in the same manner as in the coli fermentation.

#### AEROGENES TYPE OF GLUCOSE FERMENTATION (MAIN END PRODUCTS UNDERScoreD)



Hydrogen arises only from the decomposition of formic acid while carbon dioxide comes both from this source and also from the decarboxylation of pyruvic acid in the course of acetylmethylcarbinol formation. A general understanding of these different pathways of fermentation is of considerable help in the interpretation of the common laboratory tests for the biochemical differentiation of these closely related, gram-negative enteric bacteria. A scheme to illustrate the main course of the aerogenes type of fermentation is presented on page 227.

**Miscellaneous Reductions.** In the fermentations considered in the preceding pages organic compounds were the final hydrogen acceptors in the oxidations occurring during dissimilation. Inorganic compounds may also act as hydrogen acceptors in anaerobic respiration, and reduced inorganic matter accumulates in the culture medium. The most common example is that of nitrate reduction in broth cultures, a reaction which has long been employed in the laboratory as one of the more common biochemical differentiation tests. Some bacteria reduce nitrates only as far as nitrites; others carry the reduction to ammonia, while still others reduce nitrates to nitrous oxide and molecular nitrogen. This latter reaction, commonly spoken of as denitrification, is encountered at times in waterlogged soils and results in a decrease in soil fertility.

Sulfate reduction is commonly associated with species of the genus *Desulfofribrio*, which oxidize organic matter under anaerobic conditions with sulfate as the oxidizing agent, the sulfate being reduced to sulfide in the reaction. Carbonate reduction is of somewhat more general occur-



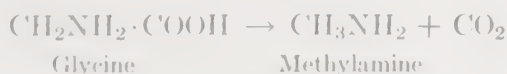
FIG. 10-7. Negatively stained preparation of a sulfate-reducing bacterium, *Clostridium nigrificans* (formerly designated as *Sporovibrio desulfuricans*). (Courtesy of R. L. Starkey.)

rence than sulfate reduction, being carried out by a variety of bacterial species wherever organic matter is being dissimilated under natural conditions and in the absence of air. Carbon dioxide acts as the final hydrogen acceptor in this type of anaerobic respiration and is reduced to methane ( $\text{CH}_4$ ), the chief constituent of the marsh gas which is frequently noted arising from the mud at the bottom of marshes and ponds. It is produced in considerable quantities in sludge-digestion tanks in sewage-disposal systems.

**Proteolytic Fermentation.** The degradations of proteins, frequently called the putrefactive reactions, are in their essential characteristics analogous to the degradations of carbohydrates which we have been considering. Protein molecules are too large to enter the cell directly, and extracellular enzymes are required to hydrolyze them to smaller, assimilable molecules. The power to hydrolyze native proteins is restricted to a few species of bacteria, amongst which we find *Clostridium histolyticum* and *C. sporogenes*, various species of *Proteus*, *Pseudomonas*, *Bacillus*, and *Streptococcus*, and other widely scattered species of bacteria. Hydrolysis products of protein degradation, particularly the peptones, are utilized much more readily, and peptones are widely employed as constituents of culture media. This group of substances apparently undergoes intracellular hydrolysis into the constituent amino acids, and the latter substances undergo various types of fermentation depending on the hydrogen-ion concentration of the medium, the nature of the amino acid, and the nature of the battery of enzymes possessed by the species.

The amino acid molecule, which can be represented by the general formula  $\text{R} \cdot \text{CHNH}_2 \cdot \text{COOH}$ , can be fermented in a variety of ways: deamination (removal of the  $\text{NH}_2$  group) with the formation of an acid, decarboxylation with the formation of an amine, a splitting of the amino acid molecule, or a combination of two or all three of the above main types. Deamination may be accomplished in a number of ways, and nine main pathways of amino acid degradation have been recognized. These can be illustrated as follows:

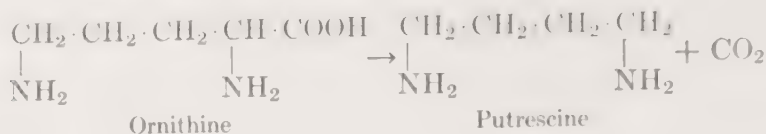
1. Decarboxylation to give an amine:



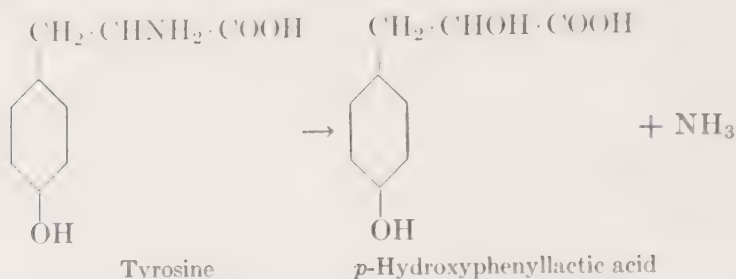
The amines are rather foul-smelling compounds and are produced by a variety of organisms under anaerobic conditions. Some of the higher amines, termed putamines, were once thought to be responsible for the symptoms of food poisoning but are now known to be relatively non-toxic when taken by way of the mouth. A typical putamine, putrescine,



can be formed by deamination of ornithine carried out by *Pseudomonas fluorescens*, *Escherichia coli*, and a variety of other bacteria. The equation for the reaction is

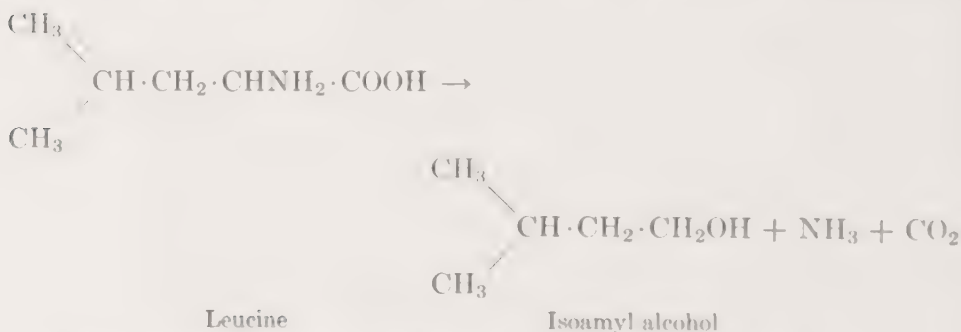


## 2. Hydrolytic deamination to give a hydroxy acid:



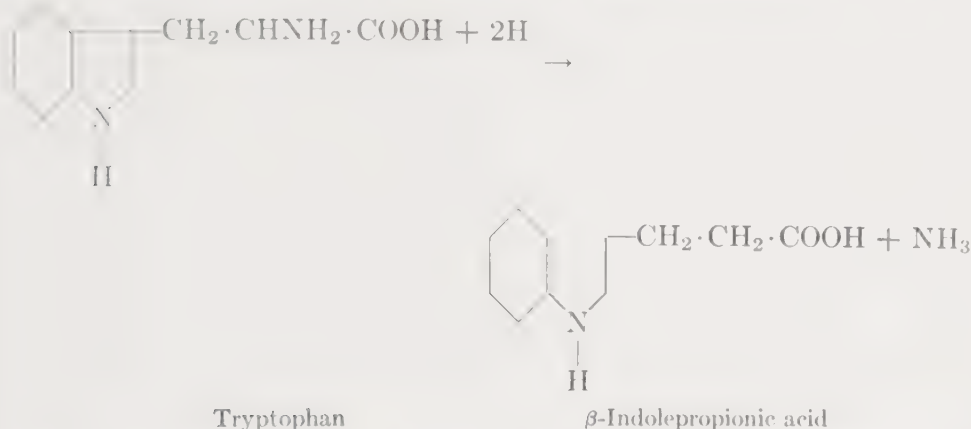
*Proteus vulgaris* deaminates tyrosine with the production of the dextro-rotatory form of the substituted lactic acid, while *Bacillus subtilis* and *Escherichia coli* ferment the *l*-form of the amino acid, giving rise to the *l*-isomer of the product. No adequate explanation has ever been advanced for the formation by one species of bacteria of an enzyme utilizing one optical isomer while another species can use only the other isomer. This holds true for all optically active foodstuff molecules.

3. Hydrolytic deamination and decarboxylation (2 and 1) to give an alcohol. This type of reaction is well illustrated by the formation of fusel oil (higher alcohols) during the course of the alcoholic fermentation of grains, certain of the amino acids present being converted into alcohols with one less carbon atom than the amino acid; e.g.,

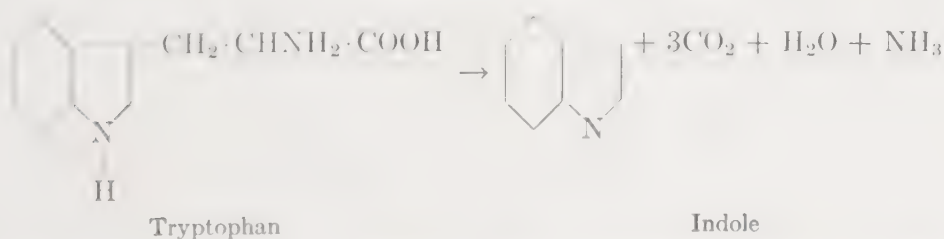


4. Reductive deamination to give a saturated acid. This type of reaction is utilized by a variety of bacteria for the oxidation of a variety of foodstuffs, the hydrogen taken up by a coenzyme being transferred

to an amino acid, which is reductively deaminated. Tryptophan, for example, is utilized as a hydrogen acceptor by *Escherichia coli* under anaerobic conditions and is converted into  $\beta$ -indolepropionic acid, or



Under aerobic conditions *E. coli* and other indole-producing bacteria attack tryptophan in a different manner, splitting off the side chain and oxidizing it to carbon dioxide, ammonia, and water, leaving indole as a waste product. Tests for indole production are commonly employed in the laboratory as an aid in the identification of bacteria.



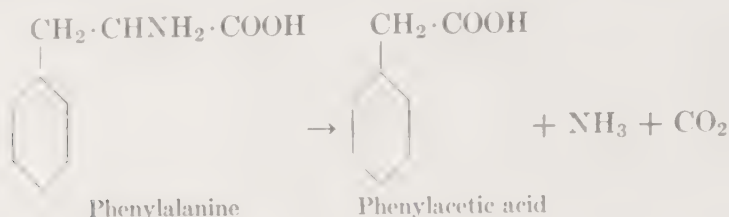
5. Reductive deamination and decarboxylation (4 and 1) to give a hydrocarbon. This type of reaction probably does not occur to any great extent. It was earlier believed that methane gas which forms in swamps, in muddy river or lake bottoms, and in sewage-disposal plants arose from this type of reaction with glycine as the substrate, or



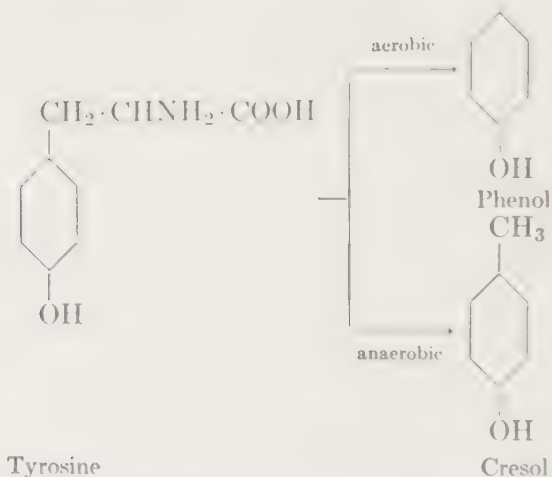
Recent studies by Barker have conclusively demonstrated that methane is formed by the reduction of carbon dioxide.

6. Oxidative deamination and decarboxylation to give an acid containing one less carbon atom than the amino acid. In this type of reaction  $\alpha$ -keto acids are commonly formed as intermediates but are usually decarboxylated to give a fatty acid and carbon dioxide. *Proteus vulgaris*

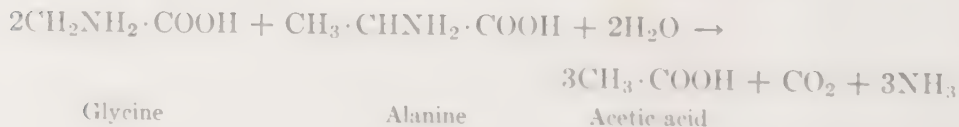
is capable of utilizing a number of the amino acids in this manner, phenylalanine being converted into phenylacetic acid:



7. Oxidative decomposition with the loss of two or more carbon atoms and the production of an acid or a hydrocarbon-like compound. This has been mentioned under 4 for the production of indole under aerobic conditions. Another interesting example of this type of reaction is the decomposition of tyrosine under aerobic conditions to give phenol, under anaerobic conditions to form *p*-cresol, both of which are toxic to the bacteria in higher concentrations than would be encountered in the culture



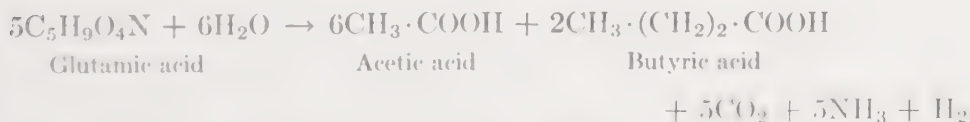
8. Coupled reactions between pairs of amino acids. This type of reaction, actually, is a combination of other reactions considered above but is characterized by the fact that one amino acid is oxidized while a different amino acid is reduced. It is frequently known as the Stickland reaction and is employed by anaerobes such as *Clostridium sporogenes* and *C. butylicum*. Glycine, for example, is reductively deaminated and alanine oxidatively deaminated and decarboxylated according to the equation



Other pairs of amino acids react in a similar manner, and there is considerable specificity as to the amino acids which react as hydrogen donors or hydrogen acceptors. *C. sporogenes* is also able to reduce a number of amino acids directly with hydrogen gas and the strong hydrogenase enzyme system it possesses.

9. Miscellaneous reactions. Sulfur-containing amino acids such as cysteine and methionine are broken down with the formation of foul-smelling mercaptans or the liberation of hydrogen sulfide. This last reaction is commonly employed in the laboratory for diagnostic purposes, sulfide formation being detected by lead or iron salts acting as indicators in the medium, reacting with sulfide to yield insoluble colored compounds. The compounds produced as a result of the liberation of hydrogen sulfide from the sulfur-containing amino acids can be dissimilated further by many species of bacteria, *Proteus vulgaris*, for example, converting cysteine to ammonia, hydrogen, carbon dioxide, and acetic acid.

Certain species of *Clostridium* ferment amino acids with the production of a variety of end products. *Clostridium tetani* produces acetic and butyric acids, carbon dioxide, and ammonia from glutamic acid, aspartic acid, or serine, and also appreciable amounts of lactic acid and ethyl alcohol with aspartic acid as the substrate. *Clostridium tetanomorphum* elicits a similar fermentation, and, in addition to the products listed above, some hydrogen is evolved. The fermentation of glutamic acid may be represented as



The source of hydrogen in fermentations of this nature is unknown, formic acid not being decomposed by clostridia of this metabolic type.

**Influence of Environmental Factors.** It is apparent that there are a considerable number of ways by which amino acids can be dissimilated by bacteria. The type of reaction which will be elicited depends not only upon the bacterium and its enzymic content but also upon environmental conditions. The degree of aeration controls to a considerable extent whether deamination will be oxidative or reductive in character, and the pH of the culture medium markedly influences the development of particular enzymes. For example, Gale has shown that *Escherichia coli* cultivated in the presence of amino acids in an acidic medium produces specific decarboxylases to a greater extent than the corresponding deaminase. Under alkaline conditions the reverse holds true. He explains this on the basis of an intracellular neutralization mechanism. In an acidic medium the amino acid is decarboxylated to yield an amine



more alkaline in character than the amino acid from which it was derived, and the production of this enzyme is of value for neutralization of the original acidity. Deamination occurs in an alkaline environment with the liberation of ammonia and the formation of an acidic compound. In other cases the cells react to an acidic environment with the formation of neutral products, *Aerobacter aerogenes* producing less acid and more acetylmethylcarbinol from glucose as the acidity of the medium increases. This neutralization mechanism, therefore, is not one peculiar to the utilization of amino acids alone.

It is apparent that the pathway of metabolism is not always the same for the same species utilizing the same substrate. Changes in the nature of the environment in which the cells are cultivated are reflected in the actual enzymes produced or in the amount produced, growth at an unfavorable pH often resulting in enhanced enzyme production to compensate for decreased activity per enzyme molecule. These changes or adaptations of the cell to its environment can take place only to a limited extent which is dependent ultimately upon the genetic composition of the species. This adaptability of the bacteria, particularly saprophytic species, enables them to survive and multiply under more diverse conditions than would be possible for more fixed species.

**Recapitulation.** It is apparent that a number of different ways exist for the anaerobic dissimilation, by different species of bacteria, of glucose or other fermentable carbohydrates. The same holds true for the anaerobic breakdown of amino acids and other substrates. These differences reflect differences in the enzymatic pattern of the various bacteria not only as regards the end products of fermentation of a particular substrate but also the foodstuffs they can utilize. It was indicated that these differences frequently are employed in the identification of genera and species.

One fundamental principle, however, is apparent in all dissimilations, aerobic or anaerobic. An organism must obtain energy to survive, to grow, and to multiply. Energy is obtained primarily through oxidation reactions in chemosynthetic organisms, energy transfer being mediated primarily by the ATP system. An oxidation involves a concomitant reduction, which means that there must be available some substance which can act as an acceptor of the hydrogens and electrons coming from biological oxidations, whether they be aerobic or anaerobic in character. The acceptor is oxygen under aerobic conditions, something other than oxygen under anaerobic conditions. Four general sources of hydrogen-electron acceptors are available, depending upon the enzymatic constitution of the species, many species being able to use two or more sources of material, depending upon conditions. These sources of an oxidant, i.e., a hydrogen-electron acceptor, can be summarized as follows:

1. Oxygen from the air or dissolved in the medium.
2. An organic compound or compounds derived from the substrate undergoing dissimilation, e.g., pyruvic acid derived from glucose and serving as the final acceptor in the lactic acid fermentation.
3. An organic compound or substance derived therefrom which must be supplied in the medium in addition to the substrate that is being dissimilated and/or oxidized, e.g., the amino acid which is reduced in the Stickland reaction.
4. Inorganic substances other than oxygen, e.g., carbon dioxide, nitrates, or sulfates for the bacteria mentioned under miscellaneous reductions. The ability to use widely different oxidizing agents, which is possessed by many of the facultative anaerobes, enables these organisms not only to survive but also to multiply under more widely divergent environmental conditions than is possible for most chemosynthetic forms of life, which are limited to the use of oxygen as the final hydrogen-electron acceptor.

## CHAPTER 11

### GROWTH REQUIREMENTS OF BACTERIA

There is a wide variety of physiological types amongst the bacteria, their sources of energy and of carbon and nitrogen ranging from simple inorganic compounds to complex organic molecules. In addition to the above requirements, bacteria, like all living things, must have a supply of water and of various elements, particularly phosphorus, sulfur, calcium, potassium, magnesium, and iron in the form of salts. Certain organisms may have quite specific inorganic requirements. For examples, *Azotobacter* requires minute traces of molybdenum or vanadium in its enzyme systems which are involved in the fixation of atmospheric nitrogen, and *Corynebacterium diphtheriae* will grow in the presence of minute traces of iron but will not produce appreciable quantities of toxin. Toxin production increases with increase in concentration of iron in the medium over a narrow concentration range and is inhibited by still greater amounts of iron. These examples illustrate that traces of certain elements may not be involved in the actual structure of the cell but that they are important in special cellular activities. Fortunately the requirements for these trace elements are generally met in the salts and organic complexes employed by the bacteriologist in his culture media.

The types of food material that must be supplied to an organism may be considered to fall into six main groups:

1. Water
2. Regulatory substances
3. Essential ions (see above)
4. Sources of energy
5. Sources of bulk-building material
6. Accessory growth factors, vitamins, essential nutritives

Although such a classification of the constituents of a culture medium is convenient for purposes of discussion, it must be borne in mind that sharp distinctions between the groups are impossible and that one substance may at times exert two or more of the functions postulated in this classification.

While it may appear strange to classify water as a foodstuff, it does make up the bulk of the cell, 75 to 90 per cent of the total weight of

the cell being due to its water content. Water also takes part in many chemical reactions essential to the synthesis of cellular material and to the maintenance of life. Water is the most universal solvent and serves as a vehicle for the transport of foodstuff into, and of waste materials out of, the cell. In addition it has a high specific heat, thus tending to ~~dissipate the~~ heat liberated in the energy-producing reactions without too great an increase in the temperature of the cell and its environment. It is also a good conductor of heat and thus aids in the dissipation of heat energy. Hence we see that water is highly important to the cell and that in the absence of water, life does not continue.

The regulatory components of a medium serve a wide variety of purposes, their more important functions being the control of:

1. Osmotic pressure
2. Permeability of the cell membrane
3. Hydrogen-ion concentration
4. Oxidation-reduction potential of the medium

**Osmotic Pressure and Permeability.** In general, the utilization of a given substance as a food by bacteria is dependent upon two factors. One is the possession of enzyme systems which make possible the dissimilation and assimilation of and from that foodstuff. The second factor is the concentration of the foodstuff, which may vary from a lower limit below which the microbe will not grow to an upper limit beyond which additional food is simply excess and in fact may become toxic to the cell. Sugar in appropriate concentration is generally a valuable food for bacteria, but in high concentrations it is inhibitory; note the use of syrup in the preservation of fruits and fruit products. Much of the inhibitory effect of high concentrations of foodstuffs is due to the high osmotic pressure developed in the solution as compared with the osmotic pressure of the cell contents, thus resulting in passage of water out of and consequent dehydration of the latter. Too low an osmotic pressure may also be detrimental, causing an abnormal passage of water into the cell. The concentration of foodstuffs and of regulatory materials must be so adjusted that the resultant osmotic pressure is conducive to the growth of the cell. Frequently not only the total concentration of the constituents of the medium but also the relative amounts of the various ions must be taken into account, since ionic ratios can markedly alter permeability of the cell and other activities as well.

The cell membrane must permit the intake of nutrient material and the output of waste products of its metabolism, at the same time preventing the outward passage of cell substance. We have seen that under aerobic conditions acetic acid can serve as a foodstuff for a cell while ~~under anaerobic conditions~~ it can be a waste product of carbohydrate



fermentation by the same cell. The membrane therefore exerts a highly selective and adaptive permeability. The permeability is influenced to a considerable extent by the nature of the environment, particularly by the hydrogen-ion concentration, and by the nature and relative concentrations of other ions in the medium. Too high a ratio of calcium to sodium, too little potassium, or an excess of chlorides or sulfates may be inhibitory or even toxic to the cell, quite apart from any inhibitory osmotic-pressure effect. This inhibitory action may well be due to a deleterious effect on the permeability of the cell membrane, although with organisms so minute as the bacteria the permeability of the cell membrane cannot be studied readily.

**Hydrogen-ion Concentration.** Most species of bacteria grow best in a medium which is neutral in reaction, and the composition of a medium is generally so adjusted that the pH will be close to 7 after sterilization. Some of the more fastidious parasites may fail to grow if the pH is not adjusted within rather narrow limits. The pneumococcus grows most readily near the pH of blood, approximately 7.3, and may fail to grow if the pH is below 7.0 or above 8.3. Saprophytic species usually exhibit a greater tolerance to changes in hydrogen-ion concentration than the pathogenic forms. In fact, certain species of bacteria are able to grow in solutions as acid as pH 1.0 while others survive in media as alkaline as pH 13. However, the majority of bacterial species are favored by a neutral reaction while the yeasts and molds prefer a slightly acidic environment.

Bacteria tend to change the hydrogen-ion concentration of their environment, since the products of their respiration are frequently acidic or basic in character. The species which ferment carbohydrates usually produce organic acids, while basic end products of protein fermentation may accumulate during the putrefactive decomposition of nitrogenous organic matter. In order to maintain their existence, the fermentative bacteria are usually resistant to relatively high hydrogen-ion concentrations while the putrefactive bacteria tend to be more resistant to low hydrogen-ion concentrations, i.e., tolerant of alkaline conditions. This fact is utilized in the preservation of foodstuffs by acids. Various foods are preserved by lactic, acetic, and propionic acids produced as a result of fermentations induced by bacteria normally occurring on the foodstuff. The acidity developed during the course of the fermentation serves to inhibit the growth of putrefactive bacteria, and foodstuffs such as sauerkraut, pickles, or onslage so preserved remain edible for long periods of time. It is apparent that by proper adjustment of the pH of a culture medium, it is possible to control to some extent the type of bacteria which will grow, or at least predominate, in that medium.

The pH of a culture medium is generally adjusted to a definite value

by the addition of an acid or an alkali. In addition, buffer salts (particularly phosphates) are frequently added to the medium for their buffering action since they react with either acids or alkalies produced by the cell and tend to neutralize these substances, thus preventing marked changes in pH. Amino acids, or complexes thereof, frequently play a similar role owing to their amphoteric character and consequent buffer action. Sometimes buffer action is obtained with the aid of a relatively insoluble salt, such as calcium carbonate, which will react with the organic acids produced during fermentation and neutralize them by the production of calcium salts.

**Oxidation-Reduction Potential.** The basic characteristic of an oxidation-reduction reaction is the transfer of electrons, although for purposes of discussion we have considered the more apparent hydrogen transfers generally encountered simultaneously in biological oxidations. The ease with which a compound undergoes oxidation (or reduction) depends to a great extent upon the ease with which it donates (or accepts) electrons. This electron-escaping tendency can be measured in many oxidation-reduction systems and is expressed in values known as the  $E_h$ , or oxidation-reduction potential, of the system. Such a value expresses in electrometric terms the ratio of oxidized to reduced components of the system at a given pH together with the relative oxidizing or reducing intensity of the system on a scale established in comparison with the oxidizing intensity of oxygen and the reducing intensity of hydrogen. A system on this scale is theoretically capable of reducing any substance above it, or of oxidizing any substance below it, on the scale, oxygen being placed at the top and hydrogen at the bottom of the scale.

If the oxidation-reduction potential of the medium is high, oxidation can occur only if there is a component of the medium possessing a still higher potential, or oxidizing potentiality. Generally this is oxygen, and therefore only aerobic growth can occur. As the potential is lowered, more electron acceptors become available and therefore more diverse types of respiratory activity. When the potential is lowered to a sufficient extent, still other electron acceptors become available and permit of anaerobic growth, in fact, many anaerobes can be cultivated in the presence of a small amount of oxygen provided that sufficiently high reducing intensities, i.e., low  $E_h$ , prevail. The  $E_h$  of a solution can be estimated with the aid of oxidation-reduction indicators, dyes which possess one color in the oxidized state and exhibit a different color in the reduced state. Methylene blue is such an indicator and has long been used by bacteriologists as an indicator of  $E_h$ , in particular with reference to anaerobiosis. When it exhibits its blue color, it indicates that conditions are not suitable for anaerobic growth, while if it is converted to its reduced colorless form, it indicates that the medium is

markedly reducing in character, a low  $E_h$ , and probably suitable for anaerobic growth. The reduction of litmus to its colorless form is also frequently noted and is employed as an aid in the identification of certain species of bacteria, particularly in a litmus milk medium.

**The Supply of Energy and Building Materials.** The compounds which are employed as sources of energy for the cell have already been discussed in connection with the consideration of respiratory mechanisms. The autotrophic forms, other than the photosynthetic bacteria, require ammonia, nitrites, sulfur or its compounds, ferrous iron salts, hydrogen, or other inorganic matter that they can oxidize. The autotrophic bacteria exhibit a high degree of specificity with respect to the energy-providing substrate, which has no counterpart in the respiratory activities of the heterotrophic forms. The *Nitrobacter* obtain their energy for the reduction of carbon dioxide only from the oxidation of nitrites to nitrates, while the *Nitrosomonas-Nitrosococcus* group is able to oxidize ammonia only as far as nitrites. The colorless sulfur bacteria and other autotrophs are generally just as specific in their respiratory requirements although the sulfur bacteria may utilize a variety of sulfur compounds. The hydrogen-oxidizing bacteria multiply under autotrophic conditions if provided with a supply of gaseous hydrogen but can grow as heterotrophs in the absence of gaseous hydrogen.

The heterotrophs are generally able to obtain energy from the oxidation of a wide variety of organic compounds, e.g., carbohydrates, fatty acids, amino acids, alcohols, and, with some species, fats. As long as an organic compound is susceptible to oxidation, it is generally possible to find a bacterium equipped with the proper enzyme system to carry out the oxidation.

The fifth group of foodstuffs may be considered as a source of the chemical elements which in appropriate combinations make up the bacterial cell. Chief among these elements are carbon and nitrogen, which the organism is able to combine, together with oxygen and hydrogen, into a wide variety of forms. The apparent relative unimportance of the nature of the organic compounds utilized by many of the heterotrophic bacteria suggests that these bacteria possibly dissimilate these substances with the formation of one or a limited number of organic compounds which the cells employ as building blocks for the synthesis of cellular material. In fact, recent studies indicate that the main purpose of the dissimilatory processes may be the provision of these essential building blocks and of phosphate bond energy, the energy ultimately liberated in these reactions being considered as waste products in the same sense as carbon dioxide and water. It has also been suggested that carbon dioxide is reduced by the photosynthetic and autotrophic bacteria and assimilated into complex molecules from which primary building blocks can be ob-

tained within the cell for the synthesis of other cellular constituents in the same manner as indicated for the heterotrophic forms. The various bacteria and other forms of life may be more similar than they appear at first, since there is a considerable degree of unity in their biochemistry. Carbon dioxide may also be assimilated to some extent by the heterotrophic bacteria, and no growth can occur in its complete absence. *Brucella abortus*, the meningococcus, and the gonococcus frequently require an increased carbon dioxide tension for growth on first isolation from the animal body, the exact function of the carbon dioxide being unknown. Apart from its influence on bacterial growth, carbon dioxide may influence other physiological processes such as the production of hemolysins and of an enterotoxin by the staphylococci, the production of these substances being greatly enhanced by the addition of from 10 to 20 per cent of carbon dioxide to the air which is in contact with the culture.

Numerous bacteria use ammonium salts as their source of nitrogen. Not only the autotrophs but also the heterotrophs in general are able to assimilate nitrogen from ammonia, although ammonia is not capable of serving as the sole source of nitrogen for many of the heterotrophs. In most instances, amino acids will serve as nitrogen sources for the heterotrophs. Certain amino acids appear to be assimilated directly, others to be broken down into molecular units needed by the cell which the cell cannot synthesize for itself. In general the bulk of the amino acids in a medium appears to be dissimilated with the liberation of ammonia (deamination), which can then be assimilated by the cells.

A number of studies in recent years have suggested that the requirements for small amounts of a particular (essential) amino acid or acids in the diet of the more fastidious organisms are due to the inability of the organisms to synthesize the structures present in the essential amino acids. For example, freshly isolated strains of *Salmonella typhosa* will grow in a glucose-inorganic salt medium with ammonia as the main source of nitrogen if a small amount of the amino acid tryptophan is added to the culture medium. Upon continued cultivation in the presence of decreasing amounts of tryptophan, the typhoid bacilli appear to become adapted to synthesizing tryptophan at a rate sufficient for growth in the complete absence of added tryptophan, ammonia then fulfilling all the nitrogen requirements. More recent studies suggest that this is not an adaptation but rather a selection of variants (normally present) which can synthesize tryptophan. The need for the inclusion of specific amino acids in the culture medium does not appear to be entirely due to the lack of certain synthetic abilities on the part of the cell but may be due in part to other factors as well. The whole problem needs further investigation.



**Growth Factors.** In addition to the gross sources of carbon, nitrogen, and other elements required by the cell, there are numerous species which require the presence of definite molecular structures, other than the amino acids, in the medium before growth becomes apparent. The essential amino acids are arbitrarily left out of this group of building substances, since the amino acids may serve other purposes as well. These growth factors (vitamins or essential nutritives) are needed in extremely small amounts and presumably cannot be synthesized, at least at an appreciable rate, by the species. The vitamins are not employed as food but instead enter a particular structure or component, frequently an enzyme or co-enzyme structure, of the cell.

The growth-factor requirements of microorganisms were recognized in part with the observations that yeast (Wildier) or *Mycobacterium phlei* (Twort) would grow in simple media only when extracts of similar organisms were added to the medium. Later it was observed that parasitic species such as *Hemophilus influenzae* could grow on nutrient agar if the medium was enriched with fresh blood. In time, it was recognized that blood contained two growth-promoting principles, X and V, essential for the growth of the influenza organism, and these have been identified as hematin (X factor) and DPN (V factor). In more recent years, other growth factors have been isolated and identified, and satisfactory culture media of known chemical composition are now available for the cultivation of many of the more exacting species. Media of this nature, prepared from chemicals of known composition, are spoken of as *synthetic media*. A partial list of growth factors required by various species of bacteria is presented in Table 11-1. The parasitic and pathogenic species in general are the most exacting in their nutritional requirements, as evidenced in part by their need for growth factors indicated in Table 11-1. This apparently reflects a loss of synthetic abilities with development of the parasitic mode of life.

It is of interest to note that the majority of the vitamins required for bacterial growth are identical with members of the vitamin B complex essential for man. Other growth factors, such as glutamine, purines, pantoic acid, and *p*-aminobenzoic acid, are of diverse nature, and their function is not entirely understood. Certain microorganisms may require only one growth factor, other organisms two or more vitamins, in their diet, and marked variations in vitamin requirements are noted at times between strains of the same species and under varied culture conditions, particularly aerobiosis and anaerobiosis. It is of interest to note that in many instances the amount of growth obtained in a synthetic medium is proportional to the concentration of a particular growth factor required by a given species. This response (see Fig. 11-1) has been used for practical purposes in the assay of foodstuffs for particular vitamin con-

TABLE 11-1. TYPICAL GROWTH-FACTOR REQUIREMENTS OF BACTERIA \*

	Biotin	Nicotinic acid	Pantothenic acid	Pyridoxine	p-Aminobenzoic acid	Riboflavin	Thiamin	Hematin (X)	DPN (V)	Miscellaneous
<i>Acetobacter suboxydans</i> . . . . .	-	+	+	-	+	-	-	-	-	+
<i>Brucella abortus</i> . . . . .	+	+	+	-	-	-	+	-	-	-
<i>Clostridium butylicum</i> . . . . .	+	-	-	-	+	-	-	-	-	-
<i>Clostridium botuli</i> . . . . .	+	+	+	+	-	+	+	-	-	+
<i>Corynebacterium diphtheriae</i> . . . . .	+	+	+	-	-	-	-	-	-	+
<i>Hemophilus influenzae</i> . . . . .	-	-	-	-	-	-	-	+	+	+
<i>Lactobacillus lactis</i> . . . . .	-	-	+	+	-	+	-	-	-	-
<i>Neisseria gonorrhoeae</i> . . . . .	-	+	+	+	-	-	+	-	-	+
<i>Pasteurella pestis</i> . . . . .	+	+	+	+	+	+	+	+	-	-
<i>Diplococcus pneumoniae</i> . . . . .	+	+	+	-	-	+	+	-	-	+
<i>Proteus vulgaris</i> . . . . .	-	+	-	-	-	-	-	-	-	+
<i>Rhizobium trifolii</i> . . . . .	+	-	+	-	-	+	+	-	-	+
<i>Micrococcus pyogenes</i> var. <i>aureus</i> . . . . .	+	+	-	-	-	-	+	-	-	+
<i>Streptococcus lactis</i> . . . . .	+	+	+	+	-	+	+	-	-	+

\* Condensed from Peterson and Peterson, Relation of bacteria to vitamins and other growth factors, *Bacteriological Reviews*, 9, 49 (1945). Requirements listed do not apply to all strains of a species.

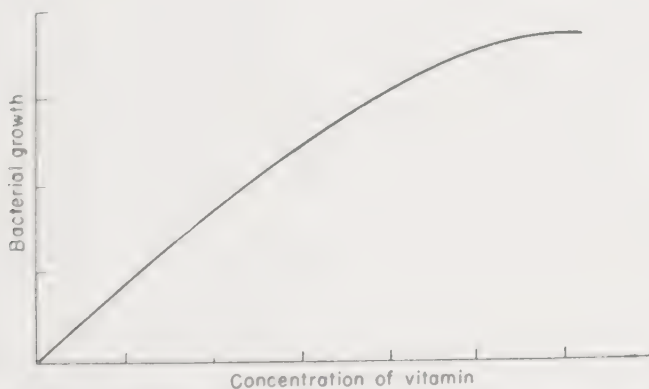


FIG. 11-1. An illustration of the influence of the initial concentration of a growth factor (vitamin or essential nutriment) on the amount of bacterial growth.

centrations and has markedly reduced the time and expense involved as compared with the older animal assay methods.

**Influence of Medium on Bacterial Flora.** It should be apparent that the nature of the food available in a given habitat will determine to a considerable extent the types of organisms which will grow and predominate in that environment. In an inorganic environment, autotrophic forms will develop, and in time sufficient organic matter may accumulate to support growth of heterotrophic forms. When carbohydrates predominate, the microbial flora will be primarily fermentative in character, while putrefactive types will tend to establish themselves in the presence of proteinaceous matter. In nature, pure cultures are seldom encountered, and the chemical transformations which occur result from the action and interaction of the members of the mixed flora. Not only the nature of the chemical components of the environment but also the concentration of these substances is important in controlling growth and the resulting flora. Within limits, the number of heterotrophic organisms which can be supported increases with increase in concentration of organic matter. But these limits vary, and one may find that bacteria grow in water supplies of low organic content and that the growth of these species is inhibited by the concentration of organic matter present in ordinary broths, although they will grow in diluted broth. There are heterotrophic bacteria which will grow in salt solutions devoid of organic matter except for traces of volatile organic matter absorbed from the air. Also the concentration of substances such as salt may markedly influence the types of bacteria which will predominate, the flora of fresh water, ocean water, and waters of high salinity being markedly different. The old saltmakers could judge when salt was about to crystallize from the evaporation ponds by the appearance of a reddish color in the water, a coloration we now know to be the result of the growth of pigmented, salt-loving forms of bacteria and algae.

All the microorganisms which we keep with great care in the laboratory as pure cultures are found in nature competing with other species and other forms of life. Nature needs the mixed activities of a varied population, while science and industry generally require specific organisms for the performance of a particular task. We can safely say that practically all kinds of microorganisms can be found more or less widespread in nature, distributed by the winds, water, or animal life. Many did not grow where they are found and would soon die out in the competitive struggle for existence. But somewhere in nature conditions are favorable for their existence and multiplication.

Grapes undergo ordinarily undergo alcoholic fermentation since yeasts are commonly present on grapes and find in the juice a relatively high sugar concentration and an acidity conducive for their growth. When the sugar supply is exhausted, organisms capable of utilizing alcohol as

a foodstuff begin to develop. Species of *Acetobacter* (acetic acid bacteria) are frequently found growing on wines and oxidize the ethyl alcohol to acetic acid, the resulting acidity generally being inimical to the growth of other microorganisms.

Raw milk on standing generally becomes acidic owing to the production of lactic acid by the lactic acid bacteria commonly encountered in milk. For a while almost pure cultures of these bacteria may predominate, but in time they are displaced by certain oxidizing yeasts or by molds. These organisms utilize or neutralize the lactic acid in sour milk, and the milk becomes alkaline in reaction. Under these conditions the putrefactive bacteria begin to grow at an appreciable rate, and the milk proteins are broken down with the formation of a variety of end products, many of which have a disagreeable odor, and the milk has definitely spoiled. Thus we see that the nature of the environment markedly influences the growth of bacteria and controls to a great extent the type of microorganism which will predominate under a given set of conditions.

**Nutrient Media.** Many different types of culture media have been employed in the study of bacteria, but most of the well-known heterotrophic species will grow in a peptone or peptone-beef extract medium, fortified if necessary with sugars or with specific growth factors. Naegeli in 1879 recognized the value of peptone as a basic constituent of culture media, and it still serves as one of the most important constituents. Proteins on hydrolysis yield simpler units known as metaproteins, proteoses, peptones, polypeptides, and finally amino acids, the products intermediate between the amino acids and the proteins being of rather indefinite chemical composition.

Peptones employed for bacteriological purposes are a complex mixture of all these products of protein hydrolysis and serve as excellent sources of carbon and nitrogen and also of certain inorganic elements for the growth of bacteria. Nutrient broth, as employed in the laboratory, is generally a solution of peptone (1 per cent) plus concentrated meat extract (0.3 per cent) and salt (0.5 per cent). Meat extract is composed of the water-soluble constituents of meat, generally beef, which will diffuse out of the meat on standing in water, and it supplements the peptone and aids the growth of the more fastidious species. Some of the more delicate pathogens grow most readily in fresh meat infusions to which peptone has been added, growth-promoting factors apparently being lost in the preparation of the extract concentrate. Extracts of other cells or tissues, vitamin preparations, blood, or other complex growth-promoting substances are frequently added to the basic nutrient broth to provide conditions suitable for the growth of the most fastidious species.

**Enrichment Cultures.** The influence of the nature of the environment was employed at a rather early date in the history of microbiology for the attempted isolation of pure species of organisms. Yeasts, relatively



free of other organisms, could be obtained from vats of wine undergoing natural alcoholic fermentation, lactic acid bacteria from sour milk, and individual pathogenic species from infected animals. When soil was added to a dilute solution of various salts with nitrites in abundance, the growth of nitrite-oxidizing bacteria was favored. On repeated transfer from one flask of this medium to another in series, the nitrite-oxidizing bacteria continued to grow while other species tended to be lost by dilution and by failure to grow under these conditions. Finally the nitrite-oxidizing bacteria, *Nitrobacter*, could at times be obtained in pure culture. When sulfur predominated in the medium, the sulfur-oxidizing bacteria tended to grow and could be obtained in pure, or at least purified, cultures. With alcohol as the substrate, simple heterotrophic alcohol-oxidizing bacteria would tend to gain the ascendancy. This technique of attempted control of growth by the selection of particular environmental conditions conducive to the growth of one type of organism is known as the *enrichment culture technique*, and the cultures themselves are called *enrichment cultures*. Taking advantage of a peculiarity of the growth requirements of a particular type of organism is not always feasible, but the enrichment culture technique is frequently employed to favor growth of the desired organism and thus to facilitate its subsequent isolation in pure culture by more modern techniques.

**Dilution Culture Technique.** As early as 1878 Lord Lister of England isolated *Streptococcus (Bacterium) lactis* in pure culture by means of a combination enrichment and dilution technique. He diluted sour milk with sterilized water and added minute amounts of various dilutions to samples of sterilized milk. The idea was that, when sufficiently diluted, some of the minute portions would contain but one cell each and the resulting growth would therefore consist of but one bacterial species. This *dilution culture technique* for the isolation of single cells, and hence of pure cultures, is quite laborious, is never too certain, and can be used satisfactorily only for the isolation of the predominant species.

**The Development of Streak and Pour Plates.** Hay infusions, meat broths, vegetable extracts, milk, urine, and other body fluids constituted the bases of the early media employed for the cultivation of microorganisms, but these substances were not particularly selective in their action. A mixed flora generally developed, and little was learned about the identity and characteristics of any particular organism, its isolation in pure culture being essential for further study of such minute beings.

Many species of bacteria cannot be differentiated one from the other under the microscope. Are they actually different? Are certain germs harmless, others helpful, still others dangerous? How can we differentiate between the different types? These are problems that confronted the early workers as well as beginning students in microbiology. Robert

Koch, a German physician, isolated the anthrax bacillus in pure culture from the blood of anthrax-infected animals, since it frequently was the only organism present in the blood stream. He cultivated *Bacillus anthracis* in pure culture in drops of sterilized liquid from the eyeballs of cattle and found that even after repeated subculturing in the laboratory, this organism retained its characteristics and upon injection into susceptible animals multiplied and produced typical anthrax infections. This bacterium, which had been observed years before by the Frenchman Davaine in the blood of sheep dying from anthrax, whetted Koch's interest in the study of bacteria themselves and in their relation to disease.

It was observed early in the nineteenth century that the red stains which at times appeared on communion wafers and on bread, particularly in the humid Mediterranean countries, were due not to the miraculous appearance of drops of the blood of Christ but rather to the growth of a red pigmented bacterium now known as *Serratia marcescens*. Ehrenberg saw these blood-red spots in 1848 and obtained growth from them on various culture media, the red color being maintained on transfer. Joseph Schroeter obtained a number of pigmented growths in 1872 on various solid media—starch and flour pastes, slices of potato, bread, and meat—and observed that bacteria in the different masses or colonies varied in appearance from colony to colony but tended to be constant in any one colony. Brefeld in 1875 reported on his methods for the isolation of single spores of fungi and on the cultivation of pure cultures from these spores in media solidified by the addition of gelatin. In 1871 Koch had succeeded in isolating certain bacteria on slices of potato, but this medium had definite limitations, and in 1881 he turned to the use of nutrient broths solidified by the addition of gelatin. The melted gelatin-broth mixture was poured on a sterile glass plate, covered with a sterile glass bell jar, and allowed to solidify. A sterilized wire was dipped into infected material and then stroked over the surface of the gelatin, the idea being that organisms would be dislodged during the streaking process and that finally the initial numbers would be so reduced that only an occasional organism would be dislodged and would thus give rise to separate colonies of individual species of bacteria. Unfortunately there are a number of organisms which will liquefy gelatin, and gelatin itself becomes fluid at body temperature, thus limiting the use of gelatin as a solidifying agent. Koch also demonstrated that the inoculum could be mixed with the liquefied gelatin before it was poured on the plate and that on cooling the gelatin solidified and held the bacteria in place, so that on incubation, discrete colonies of bacteria developed both in and on the nutrient gelatin. In this manner the *streak plates* and *pour plates* of modern bacteriology were developed. Actually

the gelatin pour-plate method was first employed by the Danish mycologist Hansen in 1880. He mixed a minute portion of yeast in a sterilized gelatin beer wort medium and spread the mixture over a cover glass. The latter was mounted on a glass ring attached to a glass slide so that an air space was provided for the growth of the yeast. The preparation could be examined under the microscope, single cells located, and pure cultures picked from the colonies developing from single cells. In 1882 the American-born wife of one of Koch's assistants, a Dr. Hesse, suggested the addition of agar-agar to nutrient broth as a substitute for gelatin as a solidifying agent. This material, derived from seaweeds, had been used in the Dutch Indies in the preparation of jellied soups and other dishes which were familiar to Mrs. Hesse. Agar proved to be an excellent substitute for gelatin as it remains in the gel state at body temperature and is attacked by very few species of bacteria. Another of Koch's pupils, Petri, in 1887 suggested the use of small covered vessels as containers for the solidified media in place of the covered glass plates. Today petri dishes are one of the most common tools of the microbiologist. Koch also introduced the use of coagulated blood serum as a solid medium for the growth of pathogenic species in 1882.

With the introduction of solid nutrient media, it became possible to isolate many species of bacteria and to grow them in pure culture. When a colony on microscopic and cultural examination appears to consist of but one species of bacteria, it can be transferred to tubes of nutrient broth or nutrient agar slants and maintained free from other species in the laboratory. On the other hand, nutrient agar is frequently inhibitory to the growth of autotrophic bacteria, and the soil bacteriologist Winogradsky introduced the use of silicic acid (water glass) as a solidifying agent for inorganic media, thus facilitating the isolation of individual species of the autotrophic bacteria.

**Illustration of Enrichment-plating Technique.** Pure cultures can be obtained in many instances most readily with the aid of a preliminary enrichment medium followed by plating out from the enrichment culture. This is well illustrated in modern methods for the bacteriological examination of water. This examination is concerned primarily with the detection of *Escherichia coli*, the presence of this organism in water serving as an indicator of possible fecal pollution of the water supply. Unless the water supply is highly polluted, this organism may be present in very limited numbers and might escape identification if a small portion of the water were plated out. Therefore, samples of the water are first added to tubes of lactose broth, the lactose favoring the growth of organisms such as *E. coli* which can use it as food. The proportion of lactose-fermenting bacteria to other species in the broth will be greatly increased on incubation, and the lactose fermenters can be more readily



detected on plating out. Substances may be added to the lactose broth to inhibit the growth of other bacteria, particularly the gram-positive forms, and such broth exerts both a *selective* and an *enrichment* action.

The enrichment culture obtained from the organisms present in the water sample is next plated out by the streak method on a medium such as Endo agar. Endo's medium consists of nutrient agar plus lactose plus basic fuchsin decolorized with sodium sulfite. Organisms which ferment lactose form aldehydes from this sugar during the course of fermentation, and the aldehydes react with the sulfite-fuchsin colorless complex, forming an aldehyde-sulfite compound and liberating fuchsin, which imparts a red color to the colony. Hence, the presence of red colonies indicates lactose fermentation, and the technician can proceed with the identification of these red colonies. A medium, such as Endo agar, which enables one to differentiate at a glance between organisms capable of carrying out a given reaction and those which are unable to bring about the reaction is known as a *differential medium*. Endo's medium, or other media based on the same principle, is also of value in the differentiation of the causative agent of typhoid fever from coliform organisms, with which it is generally associated in fecal matter. *Salmonella typhosa* and *Escherichia coli* are both short, gram-negative rods, and their colonies on nutrient agar are very similar. However, *S. typhosa* does not ferment lactose, and therefore colonies of this organism on Endo agar are colorless while those of *E. coli* are red, thus allowing rapid differentiation between the two organisms.

**Single-cell Isolation.** In certain types of work it is necessary to know not only that a given culture is composed of cells of a particular species, but that their origin can be traced back to a single cell. A culture derived from a single cell is known as a *pure-line* culture. We have seen that single-cell isolation of microorganisms started with the studies of Hansen on yeast, and that the method in brief consists in picking out one cell under the microscope and separating it from its fellows. This is a rather difficult task and can be accomplished more readily with the aid of a micromanipulator, an instrument which enables one to control accurately the movements of very small needles or pipettes in the field of vision under the microscope. In principle, the method is simple, but in practice it is quite difficult. Another method consists in selecting, with the aid of the microscope, a single, well-isolated cell on a lightly inoculated layer of nutrient agar. This cell is allowed to grow *in situ*, and cells are later picked from the colony which developed from the original cell.

**Tissue-culture Methods.** Bacteria will grow in lifeless media, and a few will grow in living tissues and produce an infection, but none of the true bacteria is restricted to growth on living tissues. We have seen



that this is one property which serves to distinguish the bacteria from the rickettsiae and filtrable viruses. These last two forms are obligate parasites and multiply only in the presence of living cells, generally being extremely specific in the type of tissue in which they will multiply. One of the most effective methods of cultivating these forms was devised by Rivers in 1931 and consists primarily in the inoculation of the parasitic agent into susceptible tissue cultivated *in vitro* by means of ordinary tissue-culture technique. The virus or rickettsia will multiply under favorable conditions and thus can be maintained and studied in the laboratory. Many bacteria will also grow under these conditions, and therefore extreme care must be employed to prevent the entrance of bacteria to the cultures of living tissues. Tissue cultures are employed in the cultivation of the poliomyelitis viruses for the production of the Salk vaccine (see Chap. 23).

One of the mysteries of biology is the susceptibility of embryonic tissue to infections against which the mature animal is highly resistant. The fluids, membranes, or tissues of partly matured chick embryos serve as an excellent pabulum for the cultivation of many microorganisms and for the growth of viruses and rickettsiae as well. Fertile eggs are readily obtained, convenient to work with, and provide a cheap but excellent source of living susceptible tissue in a natural environment. Cultures of viruses and rickettsiae in the living chick embryo are of value not only for the study of these agents but also for their production on a scale feasible for the preparation of vaccines against infections such as smallpox, yellow fever, and Rocky Mountain spotted fever. Tissue cultures and the developing egg provide the environmental conditions, and possibly even the mechanisms of respiration, for the multiplication or increase in concentration of the intracellular parasites.

**Colony Formation.** Once a cell, or group of cells, of a particular species of bacteria initiates growth on the surface of nutrient agar or other media, it will in a matter of hours give rise to a mass of many thousands or hundreds of thousands of descendants in a visible mass known as a colony. The structure and shape of a colony, which may range in size from a barely visible dot (punctiform colony) to a mass  $\frac{1}{2}$  in. in diameter, depend upon the species making up the colony, upon the particular strain of the species (see the section on the influence of variation, Chap. 14), and upon the nature of the medium. The type of colony produced on a given medium is one of the clues that a bacteriologist employs in the identification and description of a species. Size of the colony, color, optical characteristics, elevation, edge, and internal structure are important factors to consider. The latter three characteristics are studied most readily with the aid of a colony microscope. Various terms have been employed to describe these properties of colonies of bacteria. A

I. RAISED  
 II. LOW CONVEX  
 III. CRENATE  
 IV. CONVEX  
 V. CONVEX PAPILLATE  
 VI. CONVEX RUGOSE  
 VII. CONCAVE BEVELED EDGE  
 VIII. UMBONATE  
 IX. PULVINATE  
 X. ENTIRE  
 XI. EROSE  
 XII. CRENATE  
 XIII. UNULATE  
 XIV. LOBATE  
 XV. CILIATE  
 XVI. CONVEX RUGOSE  
 XVII. LACERATE  
 XVIII. RAMOSE  
 XIX. TRANSPARENT  
 XX. TRANSLUCENT  
 XXI. OPAQUE  
 XXII. FINELY GRANULAR

A = ELEVATION  
 B = EDGE  
 C = INTERNAL STRUCTURE (X 15)

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 BUREAU OF LABORATORIES  
 BALTIMORE CITY HEALTH DEPARTMENT

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The main parts that form a bacterial colony are its elevation, edge, and internal structure. Four numbers will adequately describe any colony and can be seen by reference to the chart. For example, if a colony is recorded as II, XIII, XXI, and XXIII, the colony would be one with a low convex elevation, an undulate edge, and an opaque, finely granulated internal structure. The use of numbers will

eliminate the need for long descriptive terms, and provide a quick reference system for permanent records.

It must be borne in mind that a bacterial colony will not develop to full extent unless the medium and temperature of incubation are suitable for the species under examination. The colony to be described should be well separated from other colonies on the plate to prevent interference with its development by the presence or activities of neighboring colonies. At times, marked variation in colony structure is observed within a colony, as dissociation occurs, or between different colonies of the same species. Under normal conditions, however, colony form and structure are fairly constant characteristics of a species and are of aid in its identification.

#### REFERENCE

- "Difco Manual of Dehydrated Culture Media and Reagents," Difco Laboratories, Detroit, 1948. A description of the composition of culture media, of their uses, and of the growth responses of different species together with reagents for bacteriological and serological procedures.

## CHAPTER 12

### THE MULTIPLICATION OF BACTERIA

We have been discussing growth requirements of bacteria but have not actually considered what is meant by the term growth. In ordinary usage, growth commonly implies increase in size, but when the bacteriologist speaks of growth of bacteria, he usually means population growth, i.e., increases in numbers of bacteria. A more scientific expression would be bacterial multiplication, but the term growth has so long been applied to increase in bacterial population that it has become a habit. We should bear in mind that at times not only do bacteria increase in number but the individual cells may actually increase in size, a phenomenon frequently noted in relatively young cultures.

Numerous chemical reactions are involved in the true growth and multiplication of bacteria. From comparatively simple substances in the culture medium, a bacterium is able to synthesize complex carbohydrates, fats, proteins, and other constituents and to arrange these materials in a definite pattern while at the same time it increases in size to some extent and by the process of binary fission gives rise to two cells. Each cell in turn passes through a similar phase of activity, but in spite of this rather complicated series of events, reproduction under favorable conditions takes place in an orderly manner. As a result, various phases of growth can be observed and mathematical formulas applied to express growth rates in numerical values.

**Determination of Numbers of Bacteria.** Since growth of bacteria is generally expressed in terms of numbers of bacteria, it is necessary to be able to determine the numbers of bacteria present at different times. The number of viable organisms is ordinarily determined by cultural methods, while the total number of bacteria is determined directly by microscope counts or indirectly by determinations of the total weight of the cells, of their volume, of the turbidity they impart to a suspension medium, or by the amount of chemical change they produce in the culture medium.

The number of viable cells, i.e., cells capable of multiplying in a favorable environment, is determined by cultural methods applied to appropriate dilutions of the test culture. By carrying out the dilutions, usually



in multiples of 10, in a suitable medium and observing the highest dilution in which growth occurs upon incubation, an approximation of the number of bacteria in the test culture or sample can be obtained. A large number of dilution tubes must be employed when statistically accurate results are desired. A more common method (see Fig. 12-1) is to mix

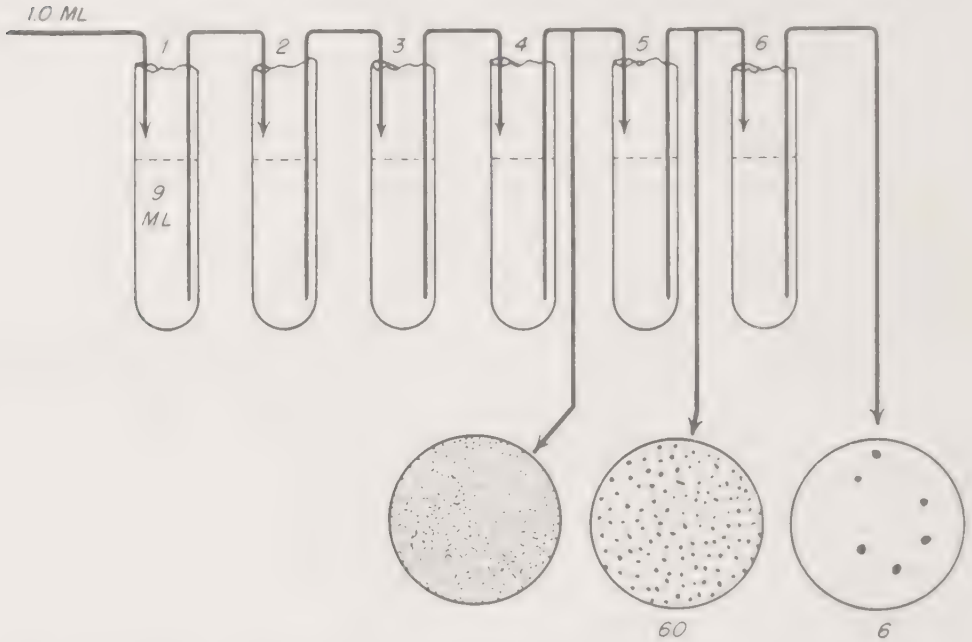


FIG. 12-1. Plate-count method for the determination of the number of viable bacteria in a culture. One milliliter of the culture is added to 9 ml. of the diluting fluid in tube 1 and the contents are thoroughly mixed. This gives a 1:10 dilution of the original culture, each milliliter containing one-tenth as many bacteria as in the original culture. One milliliter is then transferred to 9 ml. of diluent in tube 2 to give a 1:100 dilution, etc., in series. One milliliter from dilution 4 is pipetted into a petri dish, 1.0 ml. from 5 into a second dish, and 1 ml. from 6 into a third dish. Nutrient agar, melted and cooled to 50°C., is mixed with the dilutions in the plates which are then incubated for 24 to 48 hr. Counts of the number of colonies are then made. In the illustration, 1.0 ml. from tube 6, a dilution of 1:1,000,000, gave rise to six colonies. The number of colonies times the dilution ( $6 \times 1,000,000$ ) gives 6,000,000 as the number of viable bacteria in the original culture or suspension. Sixty colonies in tube 5, a dilution of 1:100,000, also gives a count of 6,000,000. Too many colonies are present in the plate from dilution 4 to obtain an accurate count.

measured dilutions of the culture with nutrient agar or gelatin in a petri dish, a count of the number of colonies developing upon incubation gives an indication of the number of viable cells present in the sample plated out. This, on multiplication by the dilution involved, indicates the number present in the original culture. Actually, not all living cells will multiply during the period of incubation (a nonmultiplying cell may still

be a living cell), and some of the colonies may represent clumps of bacteria rather than individual cells. Mechanical errors of measurement and dilution of samples are also involved, and hence the plate count does not give a wholly accurate enumeration of the live individual cells present in the material under investigation. But, if wisely employed, it is probably the most useful method for the enumeration of bacteria.

The Petroff-Hauser counting chamber, similar in construction to a blood-cell-counting chamber, can be employed for the direct enumeration of bacteria. This chamber is ruled in squares of known area and is of a known depth. Counts can be made of the actual number of cells in a definite volume of the culture or suspension. Theoretically the method appears to be excellent, but in actual practice many difficulties are encountered. Furthermore, the microscopic counts indicate the total number of bacteria, both living and dead, in the preparation, and of course a dead cell may be of little or no significance. Total numbers of bacteria can also be determined by counting the bacteria present in representative fields of stained smears prepared by spreading a definite volume of the culture or suspension over a known area. Knowing the area of the field of vision of the microscope and the total area of the smear, it is possible by simple proportion to calculate the total number of bacteria in the smear. Since the smear is assumed to contain the number of bacteria present in the measured volume of the suspension placed on the slide, one can then calculate the number of bacteria present per unit volume of the test material. Many technical difficulties are encountered in the enumeration of bacteria by this technique, but after some practice with the method fairly accurate results can be obtained. The method is frequently employed for the determination of numbers of bacteria in milk since it enables an operator to determine the relative degree of contamination of the milk in a very short period of time, cultural methods being of little value since they are time-consuming and the milk would probably be delivered and consumed before the numbers of viable bacteria in the original milk could be determined.

**Phases of Growth.** Reproduction of bacteria is primarily accomplished by binary fission, and under the most favorable conditions certain species may divide every 15 or 20 min. If this rate could be maintained for 24 hr., the progeny of a single cell would be in the neighborhood of  $1 \times 10^{21}$  cells and would have a mass of approximately four thousand tons. Cultural conditions fortunately never permit such excessive multiplication, but this high rate of multiplication does prevail for a time, as is evidenced by the rapidity with which colonies develop on agar or by the rapid increase in turbidity of a fluid culture medium.

When a culture medium is inoculated or when bacteria enter the body,

milk, foodstuffs, or anything in which they grow, they remain dormant as far as numbers of bacteria are concerned for a period of time. The duration of this period of dormancy depends on the nature of the organisms and their environment, the age of the cells, and the temperature. After a period of time, multiplication becomes evident, and the cells

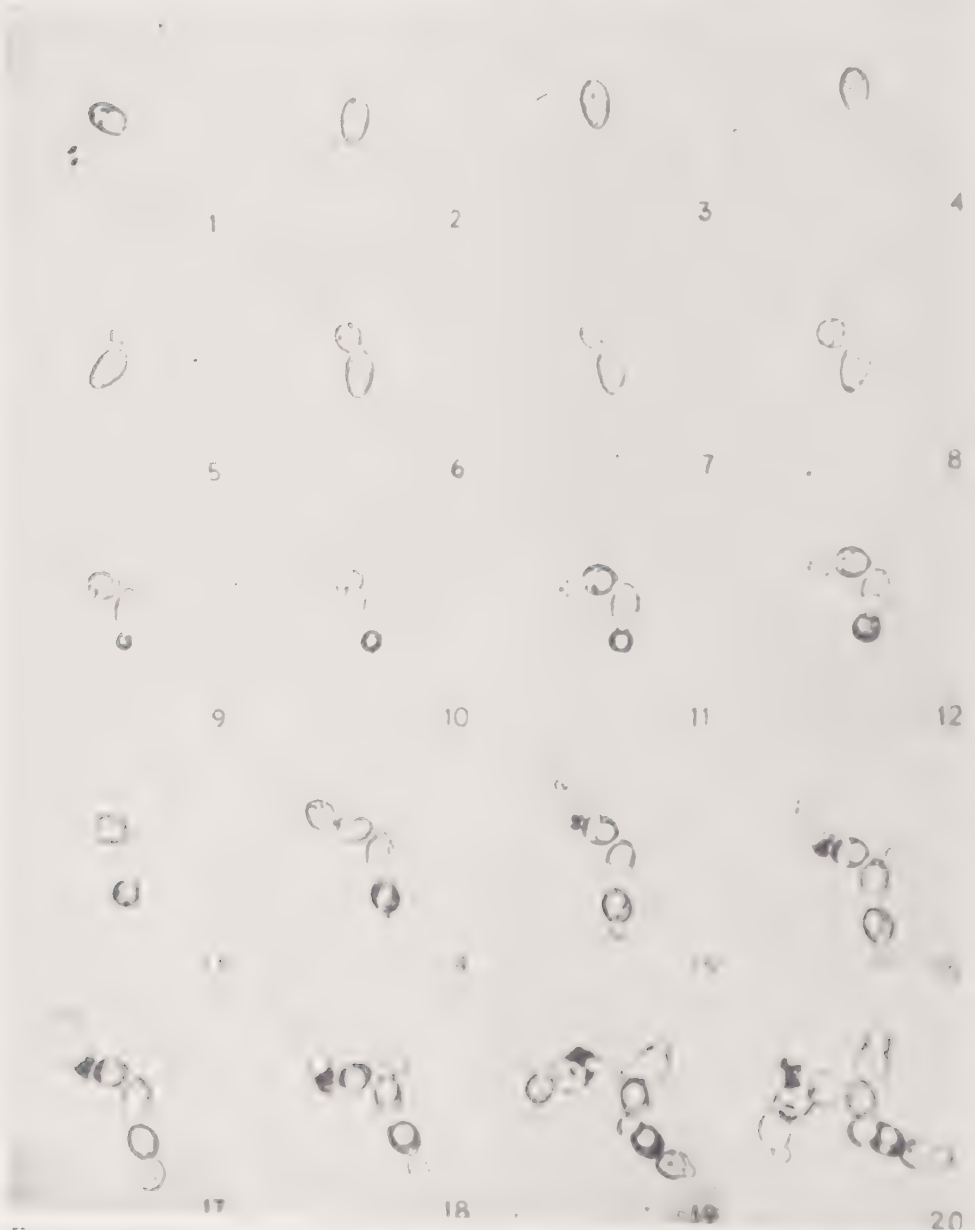


FIG. 12-2. Photomicrographic record of the growth of yeast, pictures being taken at intervals of 15 min. (From Prescott and Dunn, "Industrial Microbiology," McGraw-Hill Book Company, Inc., New York, 1949.)

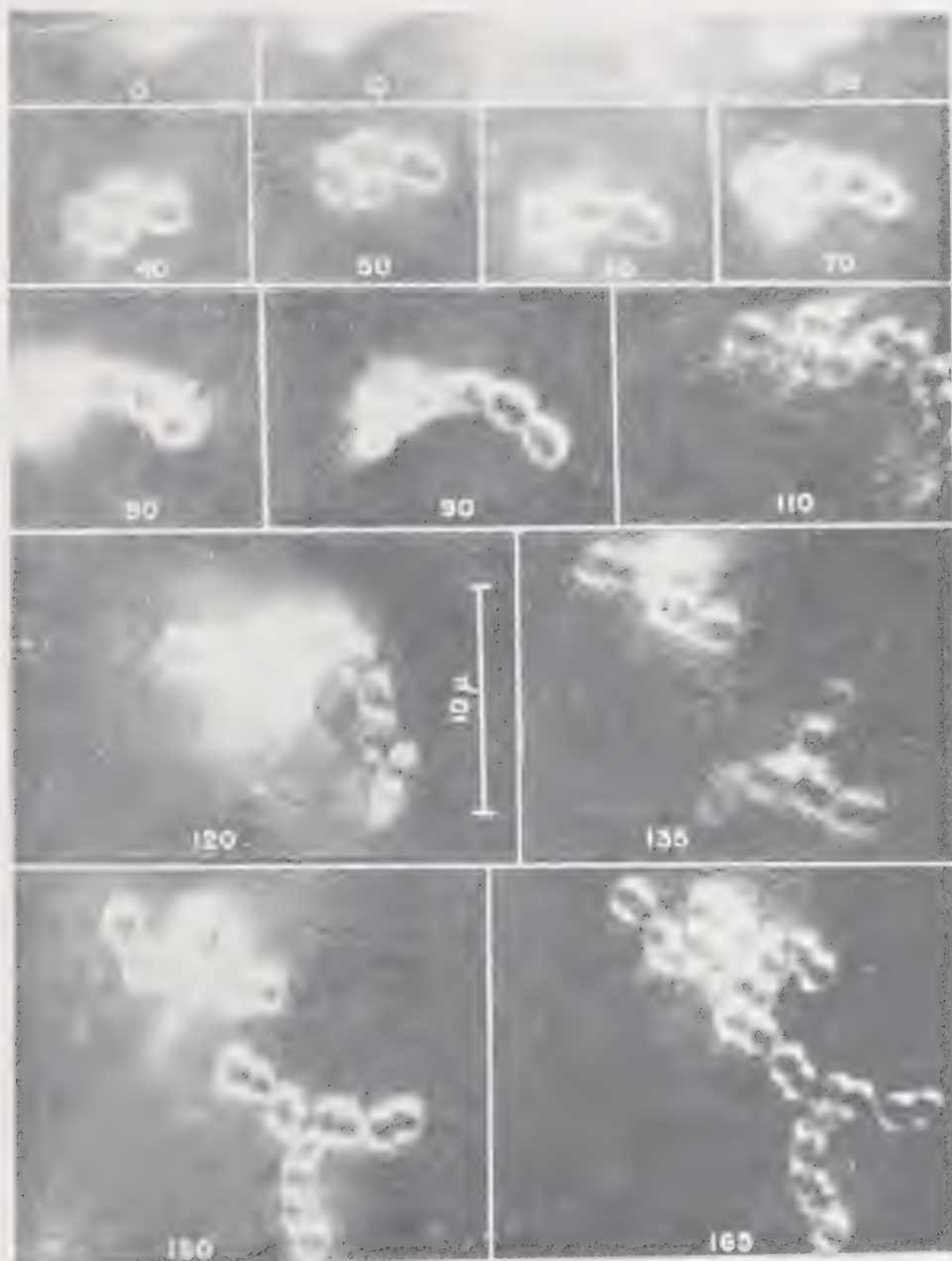


FIG. 12-3. Photomicrographic record of the growth of streptococci at time intervals (minutes) indicated. (From Knapp, "Element of Bacterial Cytology," Comstock Publishing Associates, Inc., Ithaca, N.Y., 1944.)



grow and multiply, for a time with great rapidity. Finally the rate of multiplication diminishes, in time becomes zero, and eventually the cells die. Determinations of the numbers of viable cells present at various times can be made, and when these results are plotted against time, a typical bacterial time-growth curve is obtained. Ordinarily, instead of plotting numbers of bacteria against time, we plot logarithms of the numbers of bacteria. This is done because the actual numbers can vary from one to a billion or more and would be difficult to record on a small graph, while the logarithms of the same numbers would range only from zero to nine or ten. When the logarithms of the numbers of viable bacteria per milliliter of culture medium are plotted against time in hours, a curve is obtained similar to that represented by the solid line in Fig. 12-4.

If, instead of plotting logarithms of the numbers of viable bacteria, logarithms of the total numbers of bacteria (living and dead) are plotted against time, a curve is obtained similar to that represented by the broken line. At first this curve closely parallels the viable growth curve, but as the culture ages, the two curves tend to diverge to greater extents. Only when multiplication has entirely ceased would the total cell count parallel the base line, and it would remain constant at that point until the cells begin to disintegrate. In certain species, such as the pneumococcus, destruction of dead cells by self-contained enzymes is rather common and occurs with considerable rapidity. This process of self-digestion is called *autolysis*. In other species the rate of disintegration may be quite slow and may be primarily a mechanical disintegration.

Lane-Clayton, in 1909, was the first to systematize the data on the

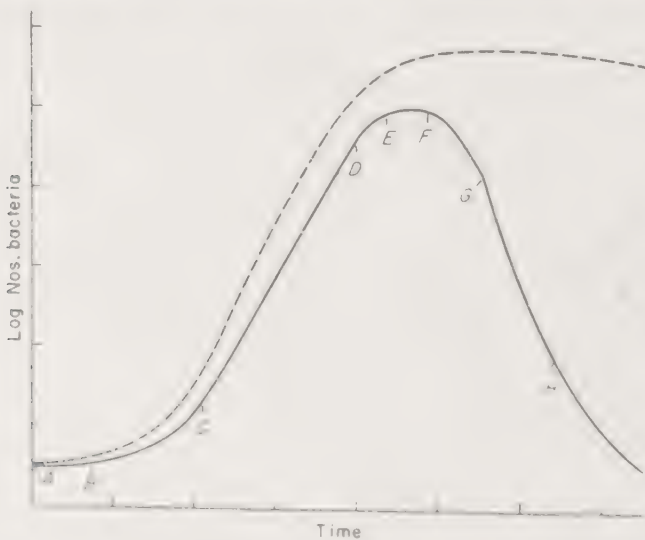


Fig. 12-4. Time-growth curve of bacterial multiplication.

various aspects of bacterial growth. She pointed out that there are four rather definite periods in the life history of a bacterial culture, as follows:

1. Lag phase, initial period of dormancy or slow growth
2. Period of regular growth
3. Stationary period, number of living cells rather constant
4. Death phase, number of living cells decreasing

Studies by Buchanan show that under favorable conditions seven quite distinct phases can be observed during the growth of bacteria. These are illustrated in Fig. 12-4 and are designated by Buchanan as follows:

1. Initial stationary phase (*a-b*). During this phase the number of bacteria remains fairly constant, and the plot is a straight line parallel to the time axis.
2. Lag, or positive-growth-acceleration, phase (*b-c*). During this phase the average rate of multiplication increases with time.
3. Logarithmic growth phase (*c-d*). During this phase the rate of reproduction per organism remains constant and therefore gives rise to a straight-line relationship.
4. Phase of negative growth acceleration (*d-e*). During this phase the rate of growth per organism decreases, but multiplication still continues.
5. Maximum stationary phase (*e-f*). During this phase there is practically no increase in the number of bacteria present; reproduction is at a standstill.
6. Phase of accelerated death (*f-g*). During this phase the number of bacteria dying per unit of time gradually increases.
7. Logarithmic death phase (*g-h*). During this phase the rate of death of the cells may be constant.

A final phase of negative acceleration in the rate of death (*h-i*) might be advantageously added to Buchanan's classification, for in many instances the death rate decreases after a time. A few cells may be very resistant and remain alive over long periods of time. This division into phases is most obvious in cultures which grow rather slowly. Henrieci reported that most organisms, when grown on a nutrient-agar surface, develop more rapidly than in liquid media, and the growth curves do not show such a sharp division into phases. The growth curve for these cultures has the appearance of a rather skewed frequency-distribution curve, showing a period of positive growth acceleration, a period of negative acceleration in growth, a period of accelerated death, and a final period of negative acceleration in death.

The previous history and amount of the original seeding and the nature of the organism and of the medium determine to some extent whether or not all of the above phases will be observed in the culture. When transplants are made in the logarithmic growth phase, the cells may continue to develop at the same rate. The initial stationary and lag phase is usually the greatest when transplants are made from an old culture and may be decreased by the use of relatively large seedings.

Winslow divided the bacterial population cycle into five general phases as follows:

1. Phase of adjustment
2. Phase of increase
3. Phase of crisis
4. Phase of decrease
5. Phase of readjustment

In a medium which is highly favorable for growth, the organisms will pass through a relatively short period of lag or dormancy with a relatively slow increase in numbers depending on the nature, age, and number of organisms in the seedling. If the medium is poorly suited for growth, there will be a decline in number of viable organisms at first, but growth will ensue after the organisms have become adjusted to the new environment.

When the phase of adjustment has been completed in a medium in which growth will occur, there next follows a period of regular and generally very rapid increase. After a lapse of time, varying with the nature of the organism, the medium, and the temperature, the period of logarithmic increase draws to a close and a phase of crisis follows. During this phase the number of viable organisms is fairly constant, the duration of the phase again being dependent on the nature of the organism and its environment. It, in some instances, lasts only a few hours and is followed by a phase of decrease or death; in other instances the phase of crisis may last for a number of days. Graphically the slope of the curve during the phase of crisis may take any form from a sharp tooth to a flat plateau.

The phase of crisis is followed by a phase of decrease in the numbers of viable cells which may follow a logarithmic relation for a time, but in time the rate of mortality decreases and the culture passes into the final phase of readjustment. If the medium is at all favorable at this time, a number of the organisms will remain alive indefinitely; in a nonfavorable medium the organisms never become readjusted and complete sterility follows. In intermediate cases there will be gradations between the one extreme and the other.

Under the most favorable conditions these phases can be further subdivided into the phases postulated by Buchanan; the same explanations apply, but Winslow's scheme is more general in type and of greater application to the general trend of bacterial growth cycles, rather than to the ideal case.

It must be remembered that increase in numbers of viable cells is taken as a criterion of growth, while actually early in the history of the culture the cells may be growing, i.e., increasing in mass and in volume,

but not multiplying. The lag period of growth may therefore be largely a result of delayed cell division.

Hemmerlén<sup>1</sup> claims that the bacterial culture cycle is of fundamental biological significance and states:

The cells of bacteria undergo a regular metamorphosis during the growth of a culture similar to the metamorphosis exhibited by the cells of a multicellular organism during its development, each species presenting 3 types of cells, a young form, an adult form, and a senescent form; that these variations are dependent on the metabolic rate, as Child has found them to be in multicellular organisms, the change from one type to another occurring at the points of inflection in the growth curve. The young or embryonic type is maintained during the period of accelerating growth, the adult form appears with the phase of negative acceleration and the senescent cells develop at the beginning of the death phase.

**Phase of Adjustment.** Numerous attempts have been made to explain the occurrence of the different phases of growth in bacterial cultures. The phase of adjustment (Winslow's classification) has been diligently studied, and three general explanations have been advanced to explain the initial lag in multiplication. These explanations can be summarized as follows:

1. *The Essential-secretion Theory.* In this theory it is assumed that an organism must secrete an essential substance into the medium before growth can take place. There seems to be some experimental evidence to substantiate this theory. Bacteria appear to exert a mutual effect, one on the other, which apparently is exceedingly important, as the lag period is generally reduced with increase in size of the inoculum. But other factors, such as the development of a suitable oxidation-reduction potential or carbon dioxide tension, no doubt play a part.

2. *The Adaptation Theory.* This theory simply assumes that a period of adaptation to the new environment is necessary before an organism can grow. No adequate explanation is offered, and seemingly this theory begs the question.

3. *The Injury Theory.* The theory held most strongly by bacteriologists appears to be the injury theory, which attempts to explain lag by assuming an injury of some sort to the parent cell. This injury may be physical or chemical in nature. Proponents of this theory suggest that as a bacterial culture ages, the cells imbibe, or perhaps fail to secrete, the toxic products of their growth. The medium surrounding the cell, as well as the interior of the cell, is saturated with this toxic substance or substances. A chemical equilibrium is established, and no movement of this material is possible in either direction. When cells so poisoned

<sup>1</sup> *Science*, **61**, 644 (1925).



are placed in a new medium where the external concentration of poison is nil, the internal inhibitory material slowly flows outward, and sooner or later the cell begins to function. The time taken for this new equilibrium to be established is the reason for the lag. In general this theory of injury repair in the fresh medium might be considered as an adaptation to new conditions. Or it may be considered that essential substances—particularly enzymes, are lost or inactivated as the cell ages and that these must be built up in appropriate concentrations before the cell is able to function to the best of its ability and to multiply.

Whatever the immediate cause of the lag phase, unquestionably the increasing growth rate is probably due to the fact that first one cell divides, then a few more, and then still more until at last a point is reached where all of the cells are dividing at a constant rate.

**Phase of Increase.** The logarithmic phase, or phase of increase, is so called because in each interval of time the majority of the bacteria are growing at a constant rate and the plot of logarithms of the numbers against time is a straight line. This log phase rarely continues for more than 5 or 6 hr. Were it to do so, we would get the enormous number of bacteria which was mentioned earlier. A great deal of work has been done concerning this phase, and while this research cannot be discussed here, a few interesting facts do stand out.

It has been shown that when subcultures are made during the log phase, there will be no lag whatever in multiplication. Sherman studied the sensitivity of the cells to toxic agents in this phase of growth and found the young cells to be more susceptible than older cells to many lethal agents. This is particularly true in the early log phase, and he considered such cultures to be "physiologically young." Henrieci observed that at the beginning of the log phase, bacteria increased in size until the middle log period was reached. Then their size diminished rapidly. Also the metabolic activities of these "physiologically young" cells may be several times greater than that of older cells (see Fig. 12-5). In general they stain more deeply and uniformly, and granules or vacuoles tend to be absent.

This question of physiological youth is not without its complications. When two bacterial cells are formed by binary fission, neither cell can be identified as parent or offspring. Are both cells to be considered as of the same age? If these two cells continue to grow and multiply, four cells are formed, again of the same age. This continues during active growth, and the result is millions of cells, all of approximately the same age. From such a consideration it could be assumed that the phenomenon of "physiological youth" should be apparent to the same degree throughout the logarithmic period of growth. Factors other than age of the cells alone must play an important role, and it should be borne

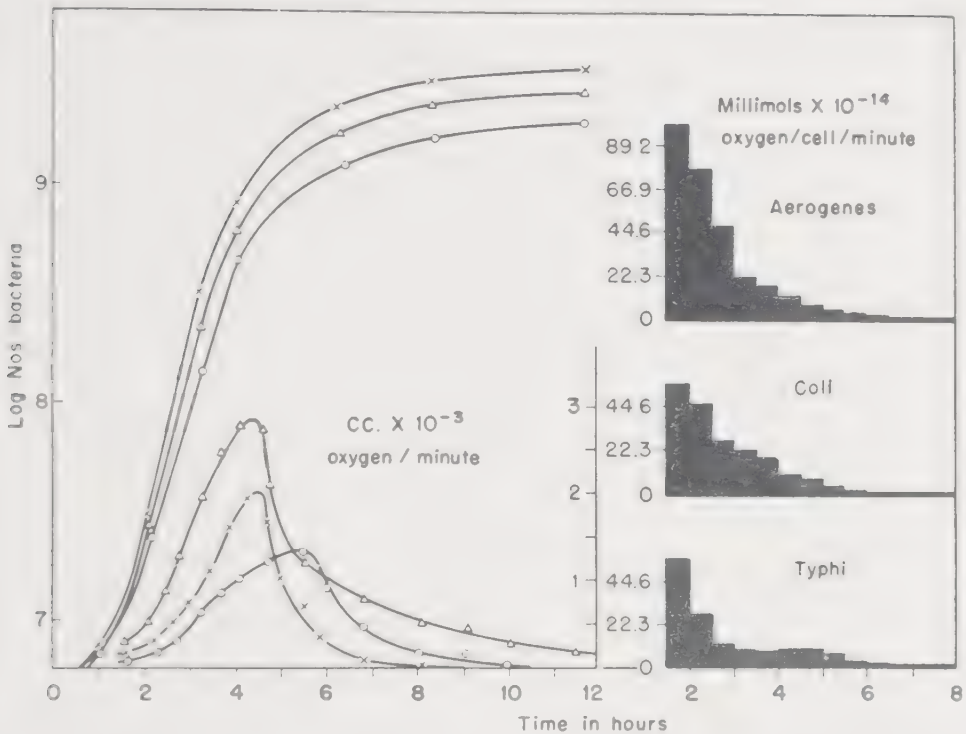


FIG. 12-5. Curves of time versus growth, versus oxygen consumption per minute per milliliter of culture, and versus oxygen consumption per cell per minute relationships observed during the growth of ( $\Delta$ ) *Aerobacter aerogenes*, ( $\circ$ ) *Salmonella typhosa*, and ( $\times$ ) *Escherichia coli* in a 1.0 per cent peptone solution at  $37.5^{\circ}\text{C}$ . [From Clifton, *Journal of Bacteriology*, **33**, 155 (1937).]

in mind that the concentration of foodstuffs is diminishing, the oxygen tension and consequently the type of respiration generally vary with time, and waste products are accumulating. Changes observed in the behavior and properties of the cells may thus in part be due to responses to a changing environment.

**Phase of Crisis.** The phase of crisis is a very interesting phase of bacterial growth, for without it the organisms would grow on and on to the exclusion of all other forms of life. This phase is the brake that Nature applies to these energetic cells, but how it works is open to question. There are some who claim that the exhaustion of food is the explanation; others think it is the accumulation of metabolic wastes or specific toxic material. Still others offer the theory of overcrowding, but this appears to be less likely when it is considered that in colonies there is crowding far in excess of that found in fluid media.

The idea that growth is limited by the accumulation of toxic substances is the one that seems to be most generally accepted, although the evidence for it is far from convincing in many instances. Unques-

tionably the accumulation of toxic substances and also the exhaustion of foodstuffs play important roles in the inhibition of the rate of bacterial growth, but which of the two is more important has not yet been determined. There is no doubt that competition for food becomes very keen in the latter part of the logarithmic phase, and as the maximum population is approached, both the amount of foodstuff available *per cell* and the amount of waste products as well must be taken into account. It must be borne in mind that the concentration of foodstuff per cell is highly important, for while there may be enough food present in the old medium *to support growth* of a small number of cells, yet there may be less than the amount required simply to *maintain* a large number of cells.

**Phase of Decrease.** Late in the period of crisis more and more of the cells die per unit time until finally they are dying at a uniform and generally most rapid rate. Considerations of the death of bacteria can be more logically postponed until we discuss the general nature of disinfection. Finally we encounter a few survivors which by some means or other have become adapted to survival in a more or less unfavorable environment, and these cells may remain viable for weeks, months, or years, depending on the nature of the cells and of their environment.

Before we leave this general discussion of phases of growth, it should be pointed out that similar periods of growth are observed in all populations, both plant and animal, under favorable conditions. In Fig. 12-6

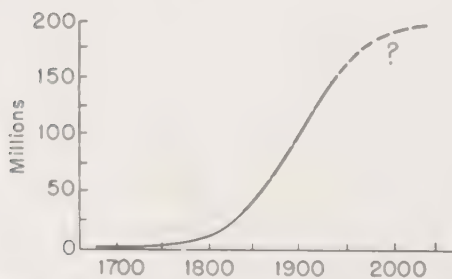


FIG. 12-6. Growth curve of the population of the United States.

is plotted the population curve of the United States. You and I help make that curve, and it resembles the growth curves for bacteria which we have been considering. Note the lag phase from 1680 to 1800, that at about this time the population began to increase at quite a rapid and uniform rate, and that we are living in this logarithmic period of multiplication. Our rate of multiplication is still pro-

portional to the population, but we are approaching a maximum population. This period of crisis has been reached in some of the crowded European countries, and in fact population decreases are being observed, even neglecting the influence of wars on populations. Bacteria are not vastly different from other forms of life!

**Mathematical Expressions of Multiplication.** For those who are mathematically inclined, equations for the calculation of generation times, number of generations, and the rate of growth of bacteria can be

developed with the aid of simple algebra. When a cell divides, it gives rise to two cells; each of these in turn on division again gives rise to two cells, or a total of four, etc., in geometrical progression, the numbers of bacteria doubling with each division time (see Fig. 12-7). This can be represented as

$$1 \rightarrow 2 \rightarrow 4 \rightarrow 8 \rightarrow 16 \rightarrow 32 \dots$$

or since

$$2^0 = 1, 2^1 = 2, 2^2 = 4, 2^3 = 8, \dots$$

we may write

$$2^0 \rightarrow 2^1 \rightarrow 2^2 \rightarrow 2^3 \rightarrow 2^4 \rightarrow 2^5 \rightarrow \dots \rightarrow 2^n$$

the exponents representing the number of generations. The same reasoning applies if we start with any number,  $B$ , of cells. At the end of a number of generations,  $n$ , there will be  $B2^n$  cells, or in other words

$$(1) \quad B_t = B_0 2^n$$

where  $B_t$  equals the number of bacteria at the end of any interval of time  $t$ ,  $B_0$  the initial number of bacteria at the beginning of the time interval, and  $n$  the number of generations in that period of time.<sup>1</sup>

Suppose we represent a generation time by  $g$ ; then

$$(2) \quad \frac{t}{n} = g$$

or

$$(3) \quad n = \frac{t}{g}$$

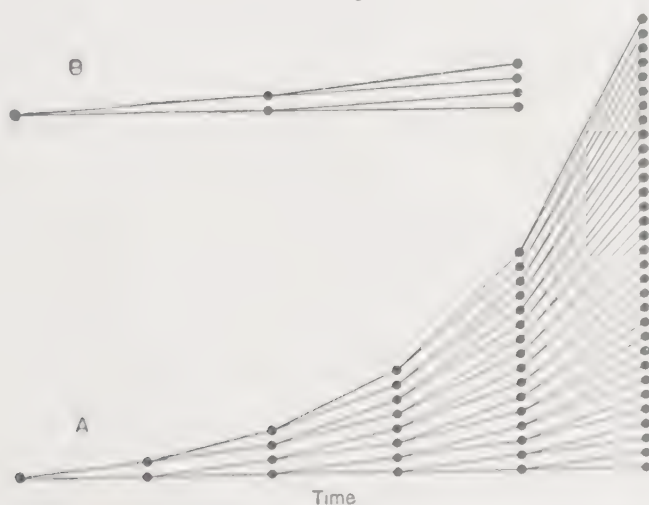


FIG. 12-7. Influence of the rate of division of bacteria on the number of cells formed per unit of time.



Many bacteria under favorable conditions divide at the rate of three times an hour or once every 20 min. In other words, there are three generations in 60 min. or

$$g = \frac{t}{n} = \frac{60}{3} = 20 \text{ min.}$$

and reasoning backward

$$n = \frac{t}{g} = \frac{60}{20} = 3 \text{ generations}$$

Equations (2) and (3) are indeed simple!

Now substitute  $t/g$  for  $n$  in equation (1), and the equation

$$(4) \quad B_t = B_0 2^{t/g}$$

is obtained. If  $B_0$  and  $B_t$  are determined in the laboratory and the time elapsed,  $t$ , is known, then these values can be substituted in equation (4) and the average generation time  $g$  can be calculated. This calculation is simplified when equation (4) is expressed in its logarithmic form

$$(5) \quad \log B_t = \log B_0 + \frac{t}{g} \log 2$$

Equation (5) can be rearranged to give

$$(6) \quad \log B_t - \log B_0 = \frac{t}{g} \log 2$$

and, on solving for  $t/g$ , we obtain

$$(7) \quad \frac{t}{g} = \frac{\log B_t - \log B_0}{\log 2}$$

On rearranging equation (7) and solving for  $g$ , we obtain

$$(8) \quad g = \frac{t \log 2}{\log B_t - \log B_0}$$

Suppose that there were 1,000 organisms at the beginning of a time interval of 60 min. and 8,000 organisms at the end of this period of time. On substitution of the logarithms of 2, 1,000, and 8,000 in equation (8), we obtain

$$g = \frac{60 \times 0.3010}{3.9031 - 3.0000} = 20 \text{ min.}$$

Now suppose that we want to obtain growth in a culture in a short period of time. We inoculate the medium with a loopful of the parent culture but

think that it is not enough so we add a second loopful of the inoculum. Will the culture develop to a definite concentration of cells in one-half the time required for a similar culture inoculated with one instead of two loopfuls of the parent cells? No. In general, doubling the size of the inoculum merely reduces the time required for a definite population to be established by one generation time,  $g$ .

When it is desirable to know the number of generations in a period of time,  $n$  can be substituted for  $t/g$  in equation (7), and we obtain

$$(9) \quad n = \frac{\log B_t - \log B_0}{\log 2}$$

On substituting the logarithms of 2, 1,000, and 8,000 in equation (9) and solving for  $n$ , we obtain the number of generations in the time period, or

$$n = \frac{3.9031 - 3.0000}{0.301} = 3 \text{ generations}$$

An equation can also be derived to represent the rate of growth of bacteria. The term rate signifies change per unit time, and the rate of multiplication, i.e., increase in numbers of bacteria per unit time ( $dB/dt$ ) is proportional to the number of cells present at that time. When this statement is expressed in mathematical terms, we obtain

$$(10) \quad \frac{dB}{dt} = KB$$

In this equation  $K$  represents the proportionality factor and is known as the velocity constant of growth.  $K$  can be evaluated from equation (10) by a process of calculus known as integration, and on integration

$$(11) \quad K = \frac{2.303}{t} \log \frac{B_t}{B_0}$$

Reverting again to simple arithmetic and assuming that at the beginning of a time period of 70 min. there are 100 cells in the culture and that at the end of 70 min. there are 1,000 cells, on substituting in equation (11), we obtain

$$K = \frac{2.303}{70} \log \frac{1,000}{100} = 0.033$$

Once the value of  $K$  is known, it is easy to calculate the number of bacteria present at any time, say 40 min. On substituting the known values in equation (11) and solving for  $\log B_t$  at 40 min. the value 2.569 is obtained. This logarithmic value is equivalent to 371 bacteria at that time.

Equation (11) strictly applies only during the logarithmic period of growth but may be applied without introducing too great an error to studies on multiplication in the latter part of the lag phase and in the early part of the phase of negative growth acceleration if the time intervals are small. As will be seen later, this equation can also be applied to the logarithmic rate of death of bacteria in cultures or to the rate of disinfection.

### FACTORS INFLUENCING RATE OF GROWTH

The conditions under which the bacteria are grown profoundly influence the growth curve. The lag period may be of short or long duration. There may be a steep or a gentle slope of the curve representing growth during the log phase. Death may be a rapid or a gradual process. Yet one can usually recognize the various phases of growth on examination of the plot of logarithms of bacteria against time. These variations are frequently due to the environmental conditions. For example, temperature, as would be expected, has a profound influence on the slope. This is obvious when one considers that at temperatures below optimum the rate of multiplication of a given species is bound to be lower than at the optimum temperature.

**Temperature.** Like all living things, bacteria have minimum, maximum, and optimum temperature requirements for growth. The majority of bacteria can withstand quite wide ranges of temperature conditions, particularly below 0°C., but the range of temperature in which they can grow and carry on their functional activities is much narrower. The general influence of temperature on the growth of a species of bacteria is well shown in a schematic illustration by Rahn, which is presented in Fig. 12-8.

For a given species the optimum temperature for growth varies with the medium, particularly if there are any inhibitory substances present; the latter will naturally exert a more pronounced effect at higher temperatures. By making a series of cultures in the same medium at different temperatures, it is possible to determine that temperature (or range) at which growth occurs most rapidly, although determinations of generation times lead to a more accurate interpretation. A minimum generation time for a given temperature does not, however, necessarily imply a maximum crop yield. In determining the optimum temperature for growth, the organisms should preferably be adapted as far as possible to growth at the various temperatures employed in the tests.

Three main groups of bacteria have been recognized according to their temperature requirements:

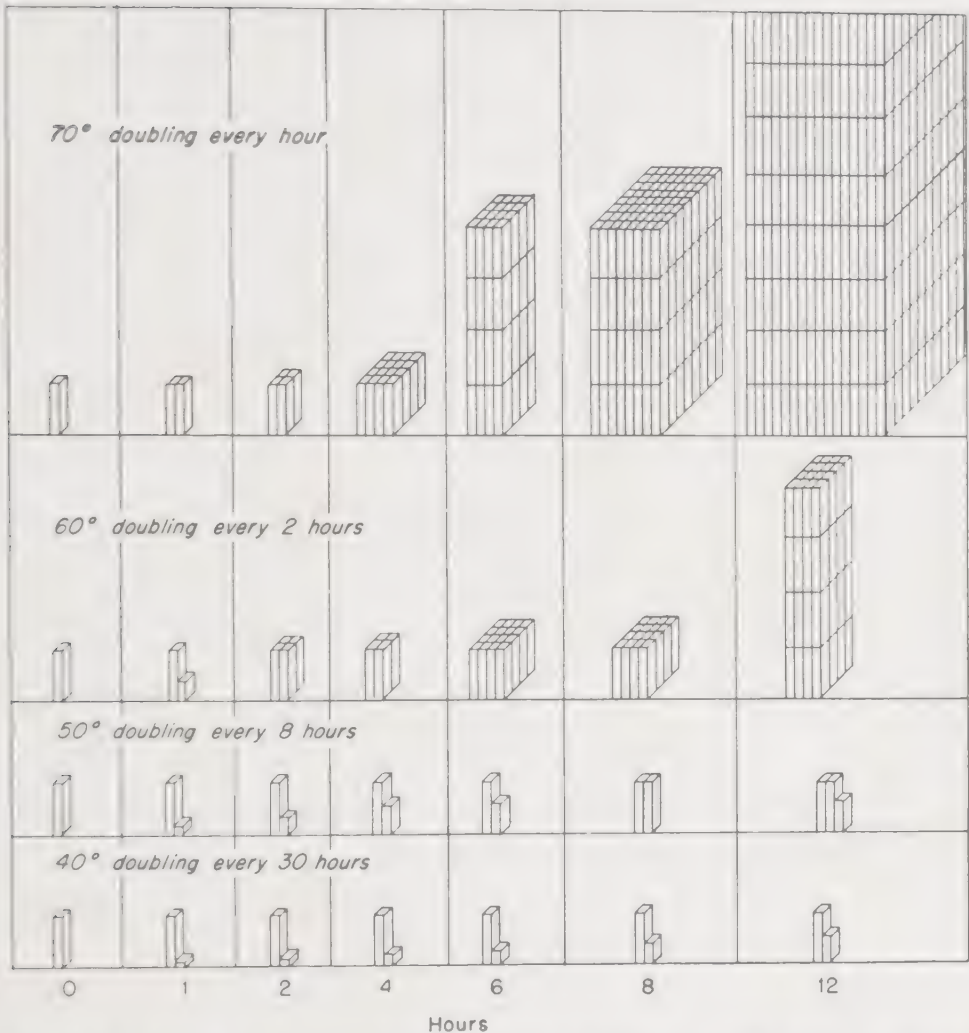


FIG. 12-8. Influence of temperature on the growth of bacteria. (From Rubin, "Microbes of Merit," The Ronald Press Company, New York, 1945.)

- 1 *Psychrophilic* (cold-loving) bacteria, usually water forms found in the depths of the ocean or other bodies of water, cold springs, and in decaying food in cold storage. The optimum temperature for growth is 10 to 20°, the minimum 0°, and the maximum 30°C.
- 2 *Thermophilic* (heat-loving) bacteria, usually found in piles of decaying organic matter or in hot springs. These bacteria often grow at a temperature as high as 80°C. Optimum growth occurs at 60 to 65°, and the minimum temperature requirement is 40 to 50°C.
- 3 *Mesophilic* bacteria, including the majority of bacteria. The minimum temperature for growth is between 5 and 25°, optimum 25 to 37°, and maximum 40 to 45°C, depending on the species. Some workers have suggested a division of the mesophilic bacteria into two subgroups as follows:



- a. *Oecophilic* bacteria, having an optimum temperature for growth at about 20°C.  
 b. *Somatophilic* bacteria, which grow best at 37.5°C. (blood heat)  
 The saprophytes and other bacteria found in the soil would belong to the first sub-group, and the forms parasitic to the warm-blooded animals fall into the latter group. There are numerous intergradations between these two subgroups, and their actual value is questionable.

Any overstepping of the maximum temperature rapidly inhibits or destroys most species, a factor of importance in pasteurization or sterilization, but the temperature requirements for growth are also of great importance in everyday life. For example, the majority of molds are unable to produce infection in man, one reason being that their upper limit for growth is frequently below body temperature. Also in butter and cheese making, tobacco curing, the wine industry, and in every industry in which bacterial action plays a role, the temperature at which the process is carried out is of great importance.

To illustrate a simple case, consider market milk stored at different temperatures. If milk is held near 0°C., a long lag phase may be observed, but finally the number of organisms will increase to many million per milliliter. No acid or marked deterioration can be detected by taste, but chemical analysis will show the presence of hydrogen sulfide and

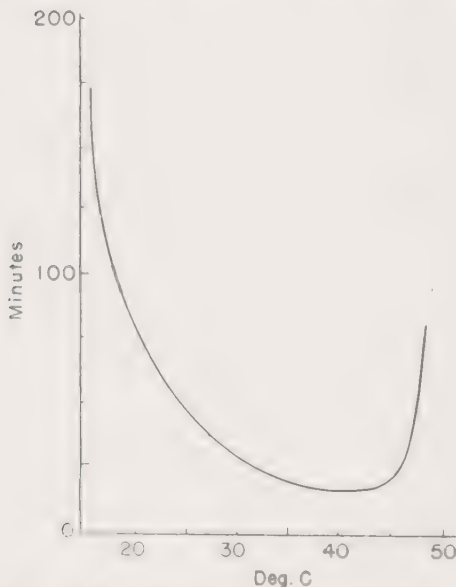


Fig. 12-9 Influence of temperature on generation time. (After Barber, *Journal of Infectious Diseases*, 5, 396 (1908).)

reaches a minimum generation time of 17 min. at 37°C., rapidly decreases above 45°, and practically ceases at 50°C. It is to be noted that the

ammonia. Between 15 and 25°C. milk sours in 36 to 48 hr., giving rise to a firm curd of an agreeable flavor without whey or gas. Near body temperature the milk sours rapidly with the production of gas and disagreeable odors, owing to the activity of organisms such as *Aerobacter aerogenes* and *Escherichia coli*. If the milk is stored at 50°C., it may keep indefinitely or, if thermophilic bacteria are present, will be decomposed with either an acid fermentation followed by digestion or by complete putrefaction, depending on the species that happens to be in the milk.

Figure 12-9 shows the effect of temperature on the generation time of *Escherichia coli*. Reproduction of *E. coli* begins at about 10°C., reaches a minimum generation time of 17 min. at 37°C., rapidly decreases above 45°, and practically ceases at 50°C. It is to be noted that the

Increase in generation time is much more abrupt above the optimum temperature than at lower temperatures.

In the range of temperature favorable for growth, an increase of 10° C. in temperature decreases the generation time by approximately one-half, an effect analogous to the doubling of the velocity of a chemical reaction for each 10°C. increase in temperature.

**Concentration of Food.** Another factor of importance in controlling the rate of growth of bacteria is the concentration of food materials. Penfold and Norris found that the rate of growth of *Salmonella typhosa* during the logarithmic phase in a 0.5 per cent saline solution of peptone at 37° C. was inversely proportional to the peptone concentration, when the latter was less than 0.4 per cent. Above this concentration further additions of peptone had little effect on the rate of growth. The addition of 0.175 per cent glucose to a 0.1 per cent peptone medium decreased the generation time by 50 per cent, but the addition of the sugar had little effect in a 1.0 per cent peptone solution. Quantitative differences were noted between samples of peptone, but the qualitative results were the same. Figure 12-10 illustrates the effect of different concentrations of peptone on the generation time. Figure 12-11 illustrates the influence of the concentration of food on the total crop yield.

The nature of the nutrient material and also the tension of oxygen and carbon dioxide are additional factors of great importance in controlling the rate of growth and total crop yield.

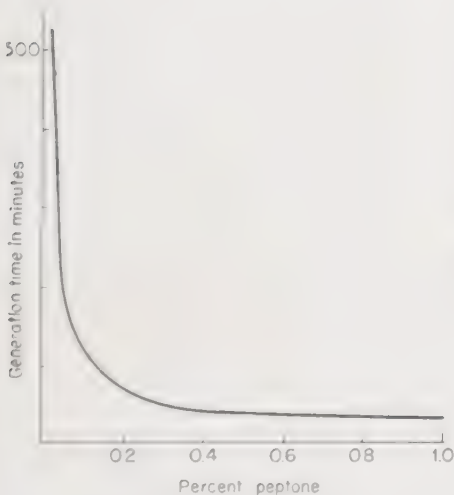


FIG. 12-10. Influence of concentration of foodstuff on generation time. [After Penfold and Norris, *Journal of Hygiene*, 12, 529 (1912).]

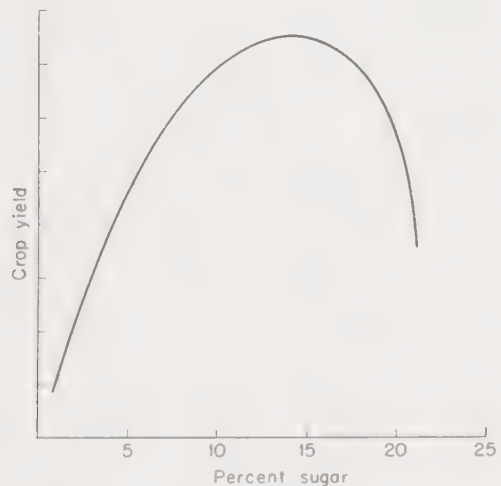


FIG. 12-11. Influence of concentration of foodstuff on the total crop yield. (After data in Rahn, "Physiology of Bacteria," The Blakiston Division, McGraw-Hill Book Company, New York, 1932.)

**Influence of Ions, Particularly  $H^+$ .** The nature and concentration of ions in the culture medium markedly influence growth and activity of bacteria. Of particular importance is the concentration of hydrogen ions. Pasteur noted in 1879 that the relatively high acidity of must favored a natural alcoholic fermentation in wine, while the low acidity of wort made the brewing of beer more difficult. He concluded that this difference in acidity determined to some extent their relative susceptibility to spoilage. He also pointed out that the acidity of the medium was a determinative factor in the establishment of lactic or alcoholic fermentation and the effective temperature for pasteurization.

The importance of controlling the acidity in bacterial culture media, in the preservation of food, in fermentation processes, in the study of enzyme activity, of disinfection, of agglutination, and of antigen-antibody reactions, and in general biological studies was realized to a greater extent with increasing studies of the bacteria. Schultz (1891) and Fuller (1895) introduced methods for adjusting the reaction of culture media by determinations of the titratable acidity and adjusting to a given "reaction value." These methods were useful, but, as pointed out by Clark, the importance of titratable acidity decreased with development of the clearer concepts of acidity and alkalinity advanced in physical chemistry. He pointed out the importance of titrating culture media to a definite hydrogen-ion concentration rather than to a reaction value. Clark and Lubs (1917), in a review of the subject, outlined colorimetric methods now generally employed for the adjustment of the hydrogen-ion concentration of culture media as well as in studies on the effect of hydrogen ions on the morphology, pigment formation, enzyme activity, fermentation, toxin production, antigenic action, electrophoresis, and viability of bacteria.

The optimal zones and limiting values of pH for bacterial growth have been investigated by many workers, and their results with typical microorganisms are summarized in Table 12-1 on page 273. The data in Table 12-2 on page 273 show the change with time in the number of viable *E. coli* in broth and in glucose broth, together with changes in pH of the media.

The pH of plain broth is at first shifted to the acid side of neutrality; as the maximum number of organisms is approached, the pH shifts towards the alkaline side, and when a pH of 8.0 is reached, there is a coincident decline in the number of viable cells.

In broth to which 1 per cent glucose had been added, the shift toward acid values of pH was more marked, the maximum population was established earlier, and death was quite rapid. It is extremely doubtful that most strains of *E. coli* would die off as rapidly as indicated by these results. In another experiment Kapnia neutralized the acid with sodium

TABLE 12-1

Organism	Acid maximum	Optimum pH	Alkali maximum
<i>Nitrobacter</i> sp. ....	3.9	6.8-7.3	13.0
<i>Escherichia coli</i> .....	4.4	6.5	7.8
<i>Bacillus subtilis</i> .....	4.2	7.5-8.5	9.4
<i>Pseudomonas aeruginosa</i> .....	5.6	6.8	8.0
<i>Aspergillus niger</i> .....	1.2	1.7-7.7	
<i>Diplococcus pneumoniae</i> .....	7.0	7.8	8.3
<i>Streptococcus pyogenes</i> .....	6.0	7.8	8.5
<i>Neisseria meningitidis</i> .....	6.5	7.5	7.8
<i>Neisseria gonorrhoeae</i> .....	6.0	7.6	8.0
<i>Salmonella paratyphi</i> .....	4.0	6.5-7.0	8.5
<i>Clostridium tetani</i> .....	5.5	7.0-7.6	8.3
<i>Corynebacterium diphtheriae</i> ..	6.0	7.3-7.6	8.2
<i>Mycobacterium tuberculosis</i> ...	5.0	7.2	8.0

TABLE 12-2 \*

Hours	Numbers in broth	pH	Numbers in 1% glucose + broth	pH
0	50,000	7.2	50,000	7.2
6	175,000,000	6.9	268,000,000	5.6
12	320,000,000	6.8	510,000,000	5.1
18	538,000,000	7.2		
24	609,000,000	7.6	221,000,000	4.8
36	559,000,000	7.9		
48	493,000,000	8.2	12,000	4.9
96	330,000,000	8.3		
192	53,000,000	8.5		
240	7,500,000	8.7		

\* From Kappas, *Scientific Reports, Government Institute of Infectious Diseases, Tokyo*, 2, 305 (1923).

hydroxide after 48 hr. when the count had fallen to 12,000 per milliliter. The number of viable organisms increased to 192,000,000 per milliliter in 12 hr. and again declined. Twice as large an increase was obtained when fresh nutrient material was added with the alkali.

Dalzell has shown that the reaction and nature of the medium are of considerable diagnostic importance. The bacterial microscopic picture of cultures from the same fecal matter, inoculated into ordinary broth,



broth of a more alkaline reaction, broth containing a large amount of bile, or the same broth acidified with 1 per cent acetic acid, is quite different. The use of only one of these mediums might give misleading results since one organism may overrun a second in a given medium, or vice versa, depending on the nature and reaction of the medium. It should also be apparent that the nature of the medium and other factors influencing growth must be carefully considered in industrial microbiological processes. For example, the substitution of a cheaper food-stuff in a culture medium for the production of yeast, provided it is readily utilized, would lower production costs. Or any alteration that would reduce the time required for growth would enable a greater production volume per unit of equipment and hence lower the cost of production.

**Ecological Considerations of Growth.** We have been considering the growth of bacteria with particular reference to pure cultures under laboratory conditions of study. Now let us briefly consider the growth of bacteria in a natural habitat such as soil, since the majority of bacteria are found in the soil. Soil can be considered to be composed of inorganic mineral particles, primarily oxides such as silicon and aluminum, and these particles are coated with a film of a colloidal system comprised mainly of organic matter. A considerable amount of air is present in the irregularities between the soil particles in a well-drained and -tilled soil. When these spaces are filled with water and the soil becomes packed, oxygen of the air can no longer penetrate with ease into the soil, and the aerobic conditions of a well-tilled soil give way to partial anaerobic conditions or to complete anaerobiosis. Fermentative decompositions then predominate, and the soil becomes "sour," to a great extent owing to the replacement of metallic ions on the soil colloids by hydrogen ions. Thus the moisture content of a soil is of importance in the control of the prevailing or prevalent bacterial flora and activities.

The layer of colloidal organic matter is analogous to a layer of agar, serving as a culture medium for the growth of bacteria and other microorganisms. Plants and animals (and their wastes) die, and their matter is contributed to the soil, thus enriching it with both inorganic and organic matter. The organic matter, chiefly proteins, fats, and carbohydrates, is broken down by microbial respiration into simpler and simpler compounds, the main end products in a well-cultivated soil being water, carbon dioxide (or carbonates), and nitrates together with traces of other salts. The process of reducing complex organic matter to salts is known as *mineralization*. These substances are then available to the green plants. Microbe growth is rapid and abundant in cultivated fields in which the organic content is kept up by manuring, is less in heavily cropped and poorly maintained soils, and in undisturbed soils

has generally reached an equilibrium. A well-cultivated rich soil might contain as many as 1,000,000,000 viable bacteria, 20,000,000 actinomycetes, and 100,000 mold fragments in addition to 200,000 protozoa and 50,000 algae in a teaspoonful of soil. The actual numbers depend upon the type of soil, moisture content, temperature, and other factors.

In the soil, or in any natural habitat, there is a continuous struggle for existence between the microbes found there. A similar struggle for existence is found between the higher forms of life, both plant and animal. In any habitat certain members of the population live in harmony with each other while all degrees of incompatibility are evident amongst others. Considerable evidence has accumulated that microbes in the soil assist one another in creating favorable environmental conditions. They also compete with each other for the available foodstuffs and at the same time may exert a variety of other influences on each other and on higher forms of life. Without all their varied activities life on earth would soon become impossible, and, as Pasteur said many years ago, "Gentlemen, it will be the microbes who say the last word."

**Mutual Relationships.** When two or more organisms live in close proximity, they may exert mutually beneficial, indifferent, or antagonistic effects. The terms *beneficial association* and *symbiosis* are employed to designate mutually beneficial relations, as contrasted to *antagonism* and *antibiosis*, which refer to a reduction in activities as a result of the living of organisms in a mixture. While many organisms do live in peaceful association, it must be borne in mind that there is a competition for food, and in a closed system one organism will in time overcrowd the other and may even kill it. Thus organisms that appear to live in peaceful association may in time show some degree of antagonistic activity.

Antagonism may be either one-sided or two-sided. In the first case, one species represses a second which is not antagonistic to it. In the other case, both organisms tend to repress each other. Under some conditions a one-sided antagonism may become two-sided while a two-sided antagonism may revert to a one-sided relationship.

It is frequently observed that when heated blood-agar plates are streaked with swabs from the throat, colonies of *Hemophilus influenzae* grow more rapidly and to a greater extent in the immediate neighborhood of colonies of *Micrococcus pyogenes*. It has been found that this phenomenon is due to the production of a diffusible substance (the V factor or DPN coenzyme) by the staphylococcus which is essential for the growth of the influenza organism and which is deficient in the blood medium. Hence an increased supply of the growth factor enhances the growth of *H. influenzae*. This relationship, which is of benefit only to *H. influenzae*, is frequently called *metabiosis*. Metabiosis in its broadest

sense is considered as the simultaneous growth of one organism on waste products of a second with no injury to the latter organism. The term is defined as a mode of life in which one organism so depends on another that it cannot flourish unless the latter precedes and influences the environment favorably. *H. influenzae* would not be dependent on *M. pyogenes* when coenzyme I is present in the medium. In the soil species of *Nitrosomonas* or *Nitrosococcus* oxidize ammonia to nitrites, and this waste product benefits *Nitrobacter* which requires nitrites as a source of oxidizable material. *Nitrobacter* species may be said to live in metabiosis with *Nitrosomonas-Nitrosococcus* species, but can we say that the latter species receive no benefit? Nitrites, which in too high concentration could be inhibitory, are removed by oxidation to nitrates, which in turn are used by the green plants. (The term commensalism is also employed for a somewhat similar relationship. Commensalism is that condition in which one organism, not truly parasitic, lives in, with, or on another, partaking usually of the same food.) Other examples of metabiosis would be the fermentation of grape juice or apple juice by yeast, which paves the way for the later development of the acetic acid bacteria, and the lactic acid fermentation of milk followed by the growth of lactic acid-oxidizing yeasts, or by molds which in turn pave the way for the growth of putrefactive bacteria.

When both members of the association benefit, the relationship is called *symbiosis*. The growth of anaerobic bacteria in the presence of aerobic forms is frequently observed. The aerobic species utilize the oxygen in the medium and thus create conditions favorable for the growth of anaerobes. The aerobes in turn may oxidize waste products of the anaerobes, and hence the relationship is reciprocally beneficial. Symbiosis between different forms of life is also frequently observed. One of the most important symbiotic relationships in the soil is that between the nitrogen-fixing rhizobia and leguminous plants. This symbiotic relationship is visually characterized by the formation of nodules or tubercles on the root systems of these plants. In these nodules the symbiotic nitrogen-fixing bacteria develop, take up nitrogen from the air, and the nitrogen becomes available in time to the plant. In return the bacteria obtain foodstuffs, and growth factors as well, from the plant. In some cases, however, the rhizobia may do marked damage to or even kill the plant, and symbiosis is replaced by an antibiotic relationship.

In the intestinal tract of animals there are metabiotic or symbiotic relationships between the bacteria and the animal. The bacteria break down complex foodstuffs into simpler molecules which can be absorbed by the animal. In turn the animal provides a continuous supply of foodstuff and an otherwise favorable habitat as well. Many animals



are unable to utilize cellulose as food since they do not possess the enzyme cellulase. Cellulose on the other hand can be utilized by a number of bacterial species, and products of its degradation will serve as food for animals. Cattle on pasturage consume considerable amounts of cellulose but are unable to digest it. It is split into simpler units by bacteria and other microorganisms living in symbiotic relationship in the alimentary tract, and these degradation products are then utilized by the cattle. Bacteriologically sterile cattle would be a most expensive source of meat! Other beneficial effects also result from symbiotic relationships in the alimentary tract. Certain microorganisms may produce an excess of vitamins, which becomes available to the animal and hence supplements the dietary vitamin supply. Vitamin requirements of animals entirely free of bacteria are in some instances greater than those of the normal animal.

*Synergism* is another term employed in the literature for a form of symbiosis. It is frequently observed that gas is produced from a given sugar by two species of bacteria growing together when neither species in pure culture produces gas from the sugar. The term bacterial synergism, or synergistic action, implies that two or more species in association produce changes which are not produced by either of the organisms alone. Synergistic action need not necessarily be a result of a true symbiotic relationship but in many instances could be the result of metabiosis (or of commensalism).

In mixed infections in plants or animals a synergistic action frequently becomes evident. For example, inflammatory reactions have been observed in which the central areas becomes gangrenous. Microscopic observation of smears from the extremities of the infected area indicated the presence of streptococci in pure culture. In the central gangrenous zone, staphylococci were also present. Apparently the streptococcus invaded the tissues, producing an inflammatory reaction and paving the way for the growth of staphylococci. The two organisms in association produced a gangrenous condition in experimental animals, but neither organism by itself was capable of producing this condition.

Relationships are not always harmonious in a microbial population, and apart from competition for food we find antagonisms developing. Antagonistic action, or *antibiosis*, is just the reverse of symbiosis. The formation of acids by fermentative bacteria inhibits the growth of putrefactive species and is an example of nonspecific antibiosis. In other instances, a specific compound may be formed by one species, and this antibiotic agent can inhibit or destroy another species. Penicillin, tyrocidin, gramicidin, pyocyanase, and streptomycin are examples of specific antibiotic agents produced by molds, bacteria, and actinomycetes, and since the organisms producing these agents are widely prevalent in the



soil, it would appear that an intense "chemical warfare" may be in continuous progress under natural conditions. In some instances even one strain of a species may inhibit growth of another (see Fig. 12-12).

Many protozoa feed upon bacteria in the soil. Higher forms feed on these simpler forms, and throughout nature we see this extreme example of antibiosis. But the depletion of any one species tends to produce

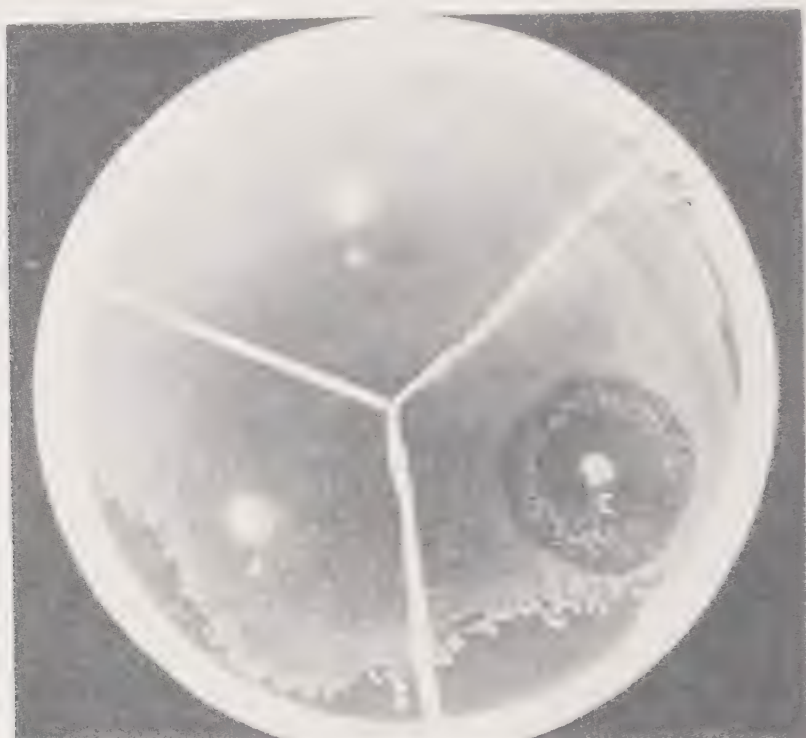


FIG. 12-12. Autoantagonism of *Escherichia coli*. Nutrient agar was spot-inoculated with three different strains of *E. coli*, and after 24 hours' incubation the plate was flooded with a suspension of strain (A). Upon further incubation the growth of (A) was not inhibited except in the vicinity of the original colony of strain (C).

renewed growth of the species, and even antibiosis may not be without mutual benefits in the long-range plans of Nature.

*Parasitism* may be considered as a mutual relationship between different species. The term parasite literally means a messmate, but in general usage usually signifies one who gets his living from another. The term *parasite* is a broad term including forms of both plant and animal life which dwell in or upon another organism called the host. Staphylococci are parasitic on the skin and mucous membranes of man and do no harm, but once they penetrate and multiply in deeper tissues, they damage the host and become destructive parasites, or *pathogens*. Para-

sitism with or without accompanying pathogenicity is widespread in nature.

In any natural habitat there is a continuous struggle for existence manifest in many ways. Generally there is a limitation of types rather than of numbers of microorganisms in natural habitats such as soil or the intestinal tract. Though a wide range of species enter these environments, many appear to be unable to survive in competition with the established forms, while at times one form may enter and establish itself at the expense of other forms. *Salmonella typhosa* may gain entrance to the intestinal tract, multiply, establish itself in great numbers in the body, and produce typhoid fever in man. Yet *S. typhosa* in another environment, soil or water, has difficulty in surviving for any length of time. Whatever the explanations, the natural habitat controls the predominating species and the rate of growth of the organisms already present or entering the particular environment. Much remains to be learned concerning the behavior of organisms in their natural environment in mixed cultures, but much can also be learned from a study of the activities of organisms in pure culture. We can hope in time to proceed from the *relatively simple* behavior of an organism in pure culture to the complex relationships and interrelationships, the *ecology*, of natural populations.

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## CHAPTER 13

### DEATH OF BACTERIA

Microorganisms are at times stimulated, inhibited, or killed by physical or chemical agents which they encounter in their environment or to which they are subjected. This necessarily holds true when they are considered to be organized groups of enzymes and other directing agents which are chemical in nature and therefore subject to the influence of other physical and chemical agents. In addition, the microbial cell possesses definite structures with specific functions, and any agent which elicits a change in these structures or their functions at the same time will be responsible for alterations in the activity of the cell as a whole. It is of importance for man to control, in so far as possible, the activities of microorganisms, whether this control is the harnessing of these cells for useful purposes or control of their detrimental activities. In this chapter an attempt will be made to summarize the general knowledge and principles concerning the control and destruction of those organisms potentially dangerous or destructive to man and his interests and to consider briefly certain of the more common agents employed for this purpose.

**Definitions.** Before discussing general principles involved in the control and primarily in the destruction of bacteria, it is necessary to define certain terms commonly employed. Considerable ignorance exists concerning the effect of physical and chemical agents on bacteria, and this has led to extensive commercial exploitation of devices or substances purported to destroy bacteria. In all fairness it should be mentioned that these agents are frequently of value for a specific purpose or under carefully controlled conditions but may become of little or no value once conditions have been slightly altered. When these agents are to be employed successfully and intelligently, one must understand what they are, the purposes for which they are intended, what factors influence their action, and just what can be expected of them. The terms usually employed for the agents intended for the control of destructive microbial action, and hence of the organisms themselves, may be defined as follows:

The term *sterilization* implies the *killing or the elimination of all forms of life* and may be accomplished by physical or chemical agents which

kill all organisms present in or on the material to be sterilized, or by their complete removal by physical means, particularly filtration. The term *sanitization*, which is employed to an increasing extent in public-health work, implies a procedure which renders material safe but not necessarily sterile.

The term *disinfectant* implies an agent employed for the destruction of microorganisms capable of producing an infection. It is synonymous with *germicide* and to a lesser extent with *fungicide*, *bactericide*, and *viricide*, the last three terms being applied to the agents utilized against the specific groups of organisms indicated by the particular names. In general the term disinfectant should be employed to represent agents intended for use away from the human host and on inanimate objects, a rather arbitrary but actually practical distinction from the term antiseptic. The killing process involved is spoken of as *disinfection*, although in common usage the term is not limited to the destruction of pathogenic species.

An *antiseptic* can be defined as an agent intended for the control of infectious agents on epithelial surfaces, on mucous membranes, or in superficial wounds. In other words it is a substance that retards or inhibits growth or activity of infectious agents but does not necessarily produce complete inhibition or death. Certain chemicals in the proper concentrations may be employed either on inert matter or on epithelial surfaces and in superficial wounds and will destroy the bacteria in the latter case with little damage to the tissues of the individual. In such a situation the distinction between disinfectant and antiseptic breaks down. The term *bacteriostatic* agent is employed synonymously with antiseptic but is broader in that it applies to the inhibition of bacteria as a whole rather than of pathogenic microorganisms alone. The term *preservative* means essentially the same as antiseptic or bacteriostatic agent but is used to denote an agent employed in food or drink to prevent spoilage by microbial activity.

The term *antinfected therapeutic agent*, or *chemotherapeutic agent*, is used to designate a substance which is designed to act after absorption into the circulating fluids. Actually a chemotherapeutic agent can be considered to be a chemical employed in therapy of any type (e.g., the use of vitamins or hormones), but in bacteriology the term is ordinarily limited to substances exerting an antimicrobial action.

The definitions considered above are not absolute ones, since the same agent might in a definite concentration act as a disinfectant, in lower concentration as an antiseptic agent, in a still lower concentration as a chemotherapeutic agent, and in still lower concentration instead of being inhibitory it might actually stimulate the microorganism. This suggests that all agents may, in appropriate concentration, be toxic



For example, sugar is a foodstuff for many bacteria, but it becomes inhibitory as its concentration is increased. In one range of concentrations it is a foodstuff, in another range, an antiseptic or preservative, and in some instances at least a feeble disinfectant or bactericidal agent. All is not so simple as the definitions imply, and therefore we must consider the various major factors involved in the destruction of microorganisms, with particular reference to the bacteria. In much of the discussion to follow, the term disinfectant will be employed in its broadest sense, that of an agent which, in suitable concentration, is capable of killing bacteria.

**Historical Development.** Early peoples observed that food could be preserved over a period of time by drying, salting, smoking, freezing, or other procedures, but they had no logical explanation for these various practices. The discovery of bacteria by Leeuwenhoek opened a new world to investigation, and he did observe that bacteria could be destroyed by heat. This was put to practical use by Appert in France in 1807 when he preserved meat or vegetables by packing them in glass containers which were stoppered and heated in boiling water. It should be mentioned that Appert apparently did not realize that heating tended to destroy organisms responsible for food spoilage but believed that heat

destroyed life-giving properties associated with air and food (see discussion of spontaneous generation, Chap. 1).

Pasteur observed that "diseases" (spoilage) of wine and beer could be greatly reduced by heating the material for a period of time sufficient to kill the bacteria, which he considered to be the cause of spoilage. This observation was followed by the development of the pasteurization process so commonly employed with milk at the present time. Pasteur's studies also led in time to the development by Lister in England of *antiseptic surgery*, infection of wounds being greatly reduced by the use of carbolic acid sprays to destroy the bacteria contaminating the wound.



FIG. 13-1. Lord Lister, originator of antiseptic surgery.

Hence, early in the history of bacteriology attempts were made to control bacteria by physical or chemical means.

These early observations were entirely practical and exploratory in nature and are of most importance in that they suggested possible methods for the control of undesirable bacteria. The second period in the study of disinfection may be said to start around 1881 with the semi-quantitative studies of Koch, in which he employed pure cultures of his test organisms and used the absence of growth in subcultures as the criterion of death. Koch observed the relatively strong action of mercuric chloride as a disinfectant, and the reputation of this material as a disinfectant and antiseptic dates from these experiments. Geppert introduced somewhat better experimental procedures in 1889 to 1891 and pointed out that the efficacy of mercuric chloride was less than had been suggested by Koch's studies, in which enough mercuric chloride was frequently transferred from the test culture to inhibit growth in subcultures. Geppert also concluded that the degree of resistance to a toxic agent varied to some extent between individuals in the same culture. At about the same time Henle recognized the importance of temperature control in experimental studies on disinfection and came to the conclusion that disinfection must be regarded as a chemical reaction between the cell and the poison and must therefore obey the laws of chemical reactions.

The studies of Kronig and Paul around 1897, of Madsen and Nyman (1907) and of Chick (1908 to 1910) introduced more quantitative methods and may be said to represent the start of modern concepts of the disinfection process. Disinfection was studied under carefully controlled conditions with a wide variety of organisms and disinfectants, and mathematical equations were employed to express the influence of various factors on the destruction of bacteria and other microorganisms. A voluminous literature on the subject of disinfection has appeared in this modern period of study. The process is so complex and so much remains to be learned that there is as yet no completely satisfactory general theory to explain the various results obtained with different organisms and different disinfectants under various test conditions. While it is not known just how bacteria are killed, yet a great deal is known concerning the major factors involved in the disinfection process, and if one remembers these factors, it is possible to use disinfectants and related agents more intelligently.

### FACTORS INFLUENCING DISINFECTION

Dead bacteria, as customarily conceived, are bacteria that do not grow when transferred to fresh nutrient agar or broth. However, this concept of dead bacteria may be fallacious in many instances, since bacteria in common with other forms of life are not necessarily dead or devoid of certain vital processes even though incapable of multiplication. Further

more, the nutrient medium employed for the subcultures may affect the results, as evidenced, for example, by studies on the destruction of anthrax spores with steam. Ten minutes' exposure to steam resulted in apparent death of anthrax spores since no growth was observed when the heat-treated spores were transferred to plain broth. In duplicate transfers to plain broth and to broth enriched with 3 per cent glucose and 5 per cent serum, growth was observed in the latter medium even after exposure of the spores to steam for 25 min. In a still richer medium even longer preliminary exposures to steam might be required to destroy any possibility of the spores reviving and germinating. As another example, cells exposed to mercuric chloride frequently do not multiply in plain broth but often do so after treatment with hydrogen sulfide or other agents which precipitate the mercuric ion either before or after the cells are placed in the plain broth subcultures. A medium containing thioglycollic acid, which reacts with mercury, is frequently employed in studies on disinfection involving the mercurials. Death is therefore partially a function of the test medium in many studies on disinfection, since cells counted as dead on one medium may be capable of multiplication in another medium.

**Concentration of Disinfectant.** It was mentioned that certain chemical agents do not influence the growth or death of bacteria unless present in excessive amounts. Other substances in relatively low concentrations may stimulate or retard growth or elicit the death of the test organism. All degrees of intergradations can be observed depending on the natures and on the concentrations of both the chemical agent and the organism, as well as upon various environmental factors. Hueppe clearly recognized this as a general biological principle in 1896 when he concluded that *every substance which in a definite concentration will kill protoplasm, inhibits development in lower concentrations, and in still lower concentrations may act as a stimulant*. This principle may be visualized, after Marshall, in the form of a disinfectant spectrum, the jagged lines indicating that no sharp line of demarcation exists between the different zones of activity evident with increasing concentrations of the agent (see Fig. 13-2). Furthermore, the width of the different bands varies with the nature of the organism and the disinfectant and with the conditions of application.

Certain substances have relatively narrow ranges of concentrations

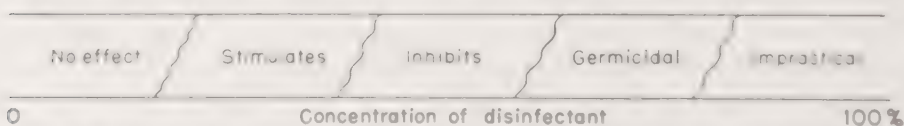


FIG. 13-2 Disinfectant spectrum. (After Marshall and Henaff, *Journal of Infectious Diseases*, 61, 44 (1937).)



which are inhibitory to growth, others wide ranges of bacteriostatic activity, and this can lead to trouble in testing the efficacy of an agent, particularly in determining when a cell is actually dead. Mathematical equations have been developed which predict the influence of the concentration of a given disinfectant on the time required for the destruction of bacteria. Without going into the mathematical details involved, one equation can be presented which represents the relation between two different concentrations,  $C_1$  and  $C_2$ , of the disinfectant and the times,  $t_1$  and  $t_2$ , required to kill an equal number of cells of the same culture. It can be written as

$$(1) \quad C_1^n t_1 = C_2^n t_2$$

in which the exponent  $n$  is a constant specific for a given disinfectant and organism under specified test conditions. It is known as the concentration coefficient and can be evaluated from somewhat complicated mathematical considerations. The derivation need not be considered here, but the practical application can be illustrated.

Substances such as phenol have a relatively large  $n$  value, which means that their activity diminishes rapidly on dilution. The time required for disinfection with phenol, which has a concentration coefficient ( $n$ ) of 4 against *Salmonella typhosa* around 25°C., can be calculated for various dilutions with the aid of equation (1). Suppose that phenol in a concentration of  $C_1$  kills all bacteria in a time  $t_1$  of 1 min. What time will be required to kill all cells when the phenol solution is diluted to a strength one-half that of  $C_1$ ? Substitution in the equation and solving for  $t_2$  gives 16 min. as the time required for disinfection to be complete. A similar calculation for phenol diluted to one-fourth the original strength indicates a disinfection time of 256 min. The time required for disinfection by a substance such as mercuric chloride with a coefficient of 1 is increased only two and four times, respectively, when the concentration is reduced to one-half and one-fourth the original strength. It is readily apparent from such calculations that some substances lose their activity as disinfectants much more readily on dilution than others, a variation which is too infrequently considered in the practical use of disinfectants.

**Time.** When a suspension of organisms is exposed to a disinfectant, not all the organisms die at once. Death, as judged by the number of viable cells remaining in the suspension, is a gradual process of measurable velocity in most instances. Generally, disinfection is considered as a process in which bacteria are killed in a reasonable length of time, but there is no agreement as to what constitutes a reasonable time for disinfection to be complete. This time is frequently determined on the basis of practicality under a given set of conditions. A consideration of the



rate of multiplication and of death of hypothetical cultures in the presence of different concentrations of a substance exerting bactericidal action permits some clarification of the concept of bactericidal and bacteriostatic action. In Fig. 13-3 increases or decreases in numbers of viable bacteria with progressing time are plotted as straight lines to simplify the discussion to follow.

The normal rate of growth is indicated in the figure by a heavy line.

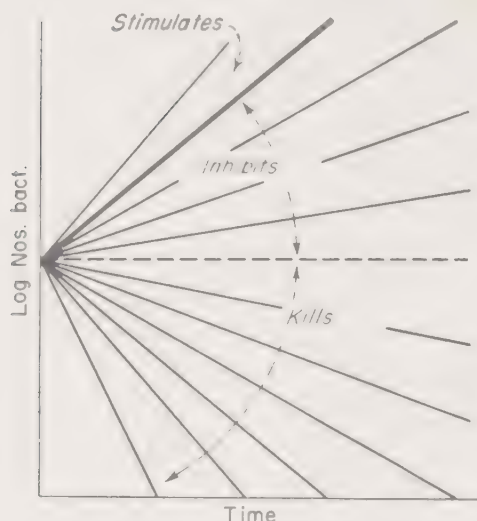


FIG. 13-3. Influence of the concentration of a chemical agent on the growth and death of bacteria. [After Marshall and Hrenoff. *Journal of Infectious Diseases*, 61, 45 (1937).]

organisms in each unit of time, and eventually a concentration is established at which death ensues in a reasonable length of time, true disinfection. Actually it is possible to consider that any rate of growth less than zero represents germicidal action while any rate greater than zero but less than normal represents bacteriostatic activity. Death of all organisms is essential, for example, in the canning industry, but the development of some degree of bacteriostasis could be worth while in the treatment of an infected wound, since a slight degree of inhibition of the bacteria might be sufficient to enable the body to cope with the invading parasite. It is also possible that higher concentrations of the disinfectant would interfere with the defense mechanisms of the body without at the same time killing all the invading organisms.

To return to a consideration of time relationships alone, it is known that in general when a particular culture is exposed to a definite concentration, generally an excess, of a deleterious chemical agent, and given

The growth rate is increased slightly in a low concentration of the agent, but once this stimulatory range has been passed, the rate of growth decreases with increase in concentration of the added agent. It is at least theoretically possible to consider that a concentration can be attained at which the rate of growth is equal to the rate of death over a period of time, these rates probably being zero. In such a case a state of complete bacteriostasis would exist, i.e., complete inhibition of growth, but at the same time no death of the organisms in the suspension during the test period. Further increases in concentration of the disinfectant naturally result in the death of some

though the organisms and the disinfectant are uniformly and thoroughly mixed, *not all the organisms die at once*. The decrease in numbers of viable organisms occurs gradually, generally the number dying during any period of time being a constant fraction of the number living at the beginning of that period. In other words, the rate of death is proportional, under a given set of conditions, to the numbers of living bacteria present at that time. In a previous chapter we considered that during the logarithmic period of growth the rate of growth is directly proportional to the numbers of bacteria present at that time. The same equation developed for the rate of growth of bacteria [equation (11), Chap. 12] applies to the rate of death with the exception that it is generally written as

$$(2) \quad K = \frac{2.303}{t} \log \frac{B_0}{B_t}$$

in which  $K$  represents the death rate or disinfection constant,  $B_0$  the number of viable bacteria at the beginning of a period of time  $t$ , and  $B_t$  the number of survivors at the end of that period of time.

Equation (2) applies quite closely to the death of bacteria in the majority of quantitative studies on the disinfection process, although some deviations are frequently observed. Let us first consider the "normal" behavior when a suspension of bacteria is exposed to a lethal concentration of a physical or chemical agent. Assume that we have a suspension containing 1,000,000 viable bacteria per milliliter and that  $K$  has a numerical value of 2.303 (for ease of calculations). On substitution of these values in equation (2) and solving for  $B_t$  at the end of 1 min., we find that there are 100,000 survivors. Similar calculations would give 10,000 at the end of 2 min., 1,000 at 3, 100 at 4, 10 at 5, 1 at 6, and 0.1 at the end of 7 min. The latter figure is not as absurd as it appears, since it is statistically significant, 0.1 survivor per milliliter meaning 1 survivor per 10 milliliters. One milliliter of a canned product might appear to be sterile on such a basis; yet there would be sufficient survivors in the can to contaminate the foodstuff. These results are plotted in Fig. 13-4 on an arithmetical scale, a semilogarithmic one, and as a distribution curve.

In the first graph in Fig. 13-4 it is apparent that disinfection is an uniformly but gradual process reaching completion as time progresses. The distribution curve indicates that a definite and constant percentage of the survivors is killed per unit time, and this is corroborated by the straight-line relationship obtained when logarithms of viable bacteria are plotted against time. While this logarithmic relationship holds in most instances, deviations are at times observed, particularly in the early and in the late

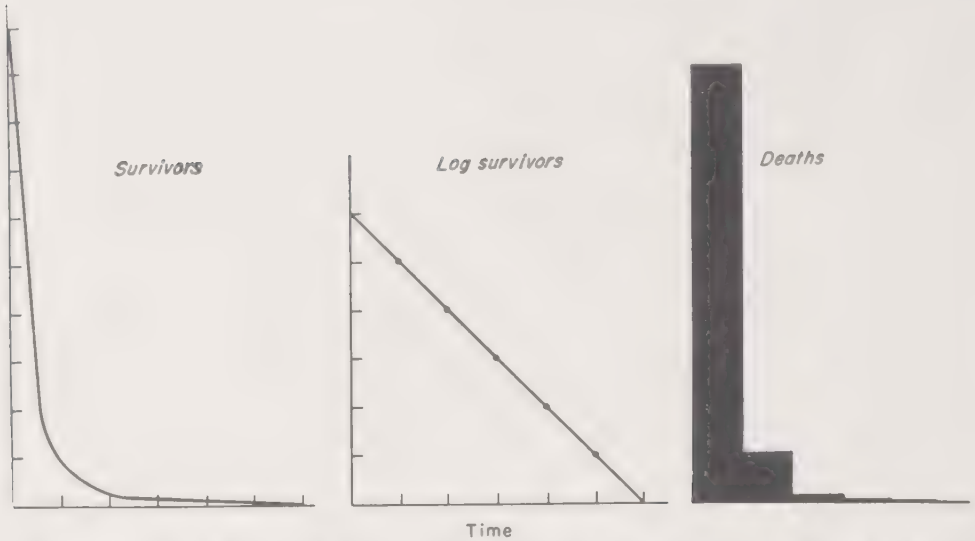


FIG. 13-4. Plots of survivors, logarithms of survivors, and numbers of cells killed per unit of time against time. The curves indicate three different ways of plotting the same data from disinfection studies.

stages of the disinfection process. One explanation is that these deviations are a reflection of differences in the resistance of individual cells, a few being highly susceptible, others highly resistant, while the majority are fairly uniform in their susceptibility to the agent. This explanation is not entirely satisfactory, but a consideration of all the major ideas concerning the discrepancies from a logarithmic death rate would lead us into discussions of an advanced nature. The main point to bear in mind is that disinfectants are not capable of producing miracles of destruction and that disinfection reaches completion under ideal experimental conditions only after a lapse of time, the length of time being dependent on a number of factors. No wonder disinfection is hard to accomplish in practice by the informed, let alone the uninformed, individual!

**Concentration of Organisms.** In ordinary practice a disinfectant is employed in considerable excess, and the concentration of organisms within reasonable limits has little influence on the time required for disinfection. Let us consider the difference in time required to reduce the number of viable bacteria to a concentration of 1 per milliliter, given two suspensions of the same organism differing only in that one suspension contains 10,000,000 and the other 1,000,000 cells per milliliter. Substituting in equation (2) and again assuming a value of 2.303 for  $K$ , we would find that there would be 1 survivor per milliliter in the first suspension at the end of 7 min., while 1 viable cell would remain at the end of 6 min. in the second suspension. Increasing the concentration of

cells 10 times only increased the time of disinfection by  $1\frac{1}{6}$  times. In actual practice the value of  $K$  may vary somewhat with the initial population, and a markedly different value for  $K$  could greatly increase the time required to kill larger numbers of bacteria. The same would hold true if the disinfectant were not employed in excess.

**Temperature.** High temperatures by themselves are lethal to bacteria, but temperature also influences the disinfection process before the lethal range is entered. At low temperatures, a  $10^{\circ}\text{C}.$  increase in temperature generally doubles the rate of a chemical reaction, and within limits the same statement holds true for the death of bacteria. In other words, with a given concentration of disinfectant the time required for disinfection is approximately halved by a  $10^{\circ}\text{C}.$  increase in temperature.

As the temperature is increased further, it by itself exerts a toxic or inhibitory action, and further increases result in death of the cells, the death rate frequently increasing to a much greater extent than doubling for each  $10^{\circ}\text{C}.$  increase in temperature. This can be expressed mathematically for a given organism under definite test conditions, but the reasoning is a bit complex and will not be presented here. It has been observed that the rate of death of vegetative bacteria around  $55$  to  $66^{\circ}\text{C}.$  may even double with each  $1^{\circ}\text{C}.$ , rather than  $10^{\circ}$ , increase in temperature. In Chick's studies, for example, it was observed that 5,000,000 typhoid bacilli could be killed in 20 min. at  $55^{\circ}$ , in 1 to 2 min. at  $60^{\circ}$ , and in 12 sec. at  $65^{\circ}$ . This lethal action of heat will be considered further in discussions on sterilization.

**Nature of the Organism.** No set rules or mathematical equations can be advanced to describe or predict the influence of the nature of the organism, of the medium, or of the disinfectant on disinfection. However, certain generalizations can be made with some degree of safety. Morphological structures such as capsules and spores, the chemical composition, and the general biological properties of the organism do profoundly influence the effectiveness of any physical or chemical agent employed as a disinfectant. Within a species the resistance to a deleterious agent can vary with the age of the culture, with its past history, and from strain to strain of the species. There are also many examples of increased resistance being established within a species on continued exposure to sublethal doses of the germicide. Certain species are frequently more susceptible than other species to a particular agent, this behavior being taken into account in the preparation of selective media. Some organisms are influenced to a greater extent than others by temperature, pH, and other changes in the environment in which disinfection is attempted. As a general rule, bacterial endospores are much more resistant to physical and chemical agents than are the parent vegetative cells. These general statements suggest that the effective use of disinfect-



ants requires an understanding of the nature of the particular organism or organisms which one wishes to destroy.

**Nature of the Medium.** It is well established that extraneous organic matter generally reduces or may even abolish the germicidal action of a given agent in a concentration which by itself would be lethal. A typical experiment indicated that the time required to kill all organisms in a bacterial suspension was 7 min. and was increased to 14, 39, and 62 min., respectively, when serum was added to give final concentrations of 10, 20, and 30 per cent. Frequently the organic matter reacts directly with the disinfectant and thereby reduces the effective concentration of the latter. In a few instances the added organic matter may exert a stimulating influence on the bacteria, which at least in part counterbalances the efficacy of the disinfectant.

Next in importance to organic matter is the acidity or alkalinity of the medium, the concentration of hydrogen or of hydroxyl ions markedly influencing the action of many disinfectants. Chlorine, for example, is a much less efficient disinfectant in alkaline solutions than in acidic ones. Acriflavine has been shown to have good antiseptic properties and fair disinfectant action *in vitro*, even in whole blood with its high organic content. It proved to be relatively inactive *in vivo*, since the circulating blood is considerably more acidic than that which has stood in the test tube. Many other examples of the influence of hydrogen ions could be cited. It must also be borne in mind that other ions can alter the efficiency of a disinfectant. The chloride ion, for example, reduces the efficacy of mercuric chloride, a common-ion effect suppressing the dissociation of mercuric chloride and reducing the concentration of the active mercuric ion. Salts too, in nontoxic concentrations, may influence the results; sodium chloride, for example, promotes the destructive action of phenol by tending to decrease the solubility of phenol in water and increasing its solubility in bacterial protoplasm. Water itself also influences the disinfection process, the lethal action of heat or of ethyl alcohol decreasing as the concentration of water is decreased.

The situation becomes even more complicated when an attempt is made to use an antiseptic concentration of a germicidal agent on living tissues. Not only do the various factors considered thus far influence the results, but here toxicity to the tissue cells must also be taken into account. Sometimes a slight amount of damage to the tissues may be a price worth paying to rid the wound of infection, but this holds true only within narrow limits.

**Nature of the Disinfectant.** It should be apparent by this time that there are no particular types of substances that can be classified as disinfectants. Probably all chemical agents can exert a deleterious action when present in appropriate concentrations. Water itself is no exception.

since many cells are disrupted when suspended in pure water. For all practical purposes a chemical agent is considered as a disinfectant if it can kill bacteria in a reasonable length of time and in a concentration of the agent suitable for practical purposes. This implies that inhibition or death of bacteria can be induced by a wide variety of agents and as a result of different types of chemical reactions.

Chemical agents active as disinfectants, antiseptics, or preservatives can be classified as (1) salts of heavy metals, (2) halogens, (3) acids and alkalies, (4) miscellaneous, and (5) organic compounds. The latter group of compounds can be subdivided in a number of ways owing to the wide diversity of molecular structures encountered in the numerous compounds of carbon.

Classification of bactericidal or germicidal agents on the basis of the nature of the substance is worth while for purposes of discussion, but classification on the basis of mode of action of the compound can be of even more value. This latter classification soon leads into difficulties, since one substance may react in more than one way and in many instances the mode of action is unknown. However, five general types of action can be suggested as follows:

1. Oxidation of cellular constituents
  - a. Halogens: chlorine, bromine, and iodine
  - b. Potassium permanganate
  - c. Hydrogen peroxide
2. Hydrolysis of cellular constituents
  - a. Strong acids and alkalies
3. Modification of the permeability of the cell membrane or of protoplasm
  - a. Unfavorable ratios of essential ions, e.g., Ca/Na
  - b. Protein-denaturing agents, such as salts of the heavy metals, alcohols, phenolic compounds, etc., and physical agents, particularly heat and light
4. Mechanical disruption by plasmolysis
  - a. Strong brines or sirups
5. Chemical union with or adsorption by vital cellular constituents such as essential metabolites, enzymes, genes, etc.
  - a. Potassium cyanide, an "iron poison"
  - b. Salts of the heavy metals. [As indicated under 3, these substances may act as protein-denaturing or -precipitating agents in general, but they can also act specifically, mercuric ions, e.g., reacting with essential sulfhydryl ( $-SH$ ) groups.]
  - c. The sulfa drugs
  - d. Antibiotic agents

It is apparent that a wide variety of physical and chemical agents can be employed for the destruction or control of microorganisms, that these substances can react in a number of ways, and that many factors influence the action of these agents. Numerous theories have been advanced to explain the modes of action of disinfectants and related agents (or concentrations thereof) and of the lethal or inhibitory processes. These are

discussed in detail in the more advanced texts and in specialized books or reviews, while we have considered only a rather generalized and somewhat chemical aspect of the situation. Until a completely satisfactory theory has been advanced, it will be necessary to base intelligent use of disinfectants upon trial methods under conditions closely related to their intended use and with reference to the general principles considered here. Most progress along such lines has been made in the use of heat as a sterilizing agent in the canning industry or as a destructive agent for disease-producing bacteria in milk, and in the physical and chemical purification of water supplies. Considerable progress is also being made at the present time in the development and use of chemotherapeutic agents, but here, as is true for antiseptics in general, not one but actually two types of organisms must be considered, the parasite and the host.

### THE EVALUATION OF GERMICIDES

The desire to express the germicidal activity of various agents on a numerical basis has led to the development of numerous procedures, most of which are based on the phenol-coefficient method developed by Rideal and Walker in 1906. Any such test enables one to list compounds in the order of their germicidal activity based upon an arbitrarily selected standard and procedure. One major drawback to such a listing is that the position of the compounds in the list will shift with alterations in procedure and in test organisms, and this shift may be very pronounced when the agent is employed under the varied conditions encountered in everyday life.

**The Phenol Coefficient.** The principles of this test remain much the same in the various procedures in use, but different organisms, test conditions, and times can be employed. Only one procedure, suggested by the U.S. Food and Drug Administration, will be outlined here. A 24-hr. culture of a particular strain of *Salmonella typhosa* is exposed under rigorously controlled conditions to the action of different dilutions of phenol and of the test agent for 5, 10, and 15 min. Transfers are made to nutrient broth at the end of each of these three intervals of time, and the subcultures are incubated for 48 hr. (see Fig. 13-5). The phenol coefficient is calculated by dividing the highest dilution of the test germicide killing the test organism in 10 min., but not in 5 min., by the corresponding dilution of phenol. Assume that a 1:100 dilution of phenol killed (no growth in subculture) in 10 min. exposure but not in 5 min., while a 1:225 dilution of the test disinfectant elicited the same results. The phenol coefficient would be  $\frac{225}{100}$ , or 2.25. All that this value actually means is that, under the conditions of the test, 2.25 times as much phenol as the test disinfectant would be required on a unit-of-mass

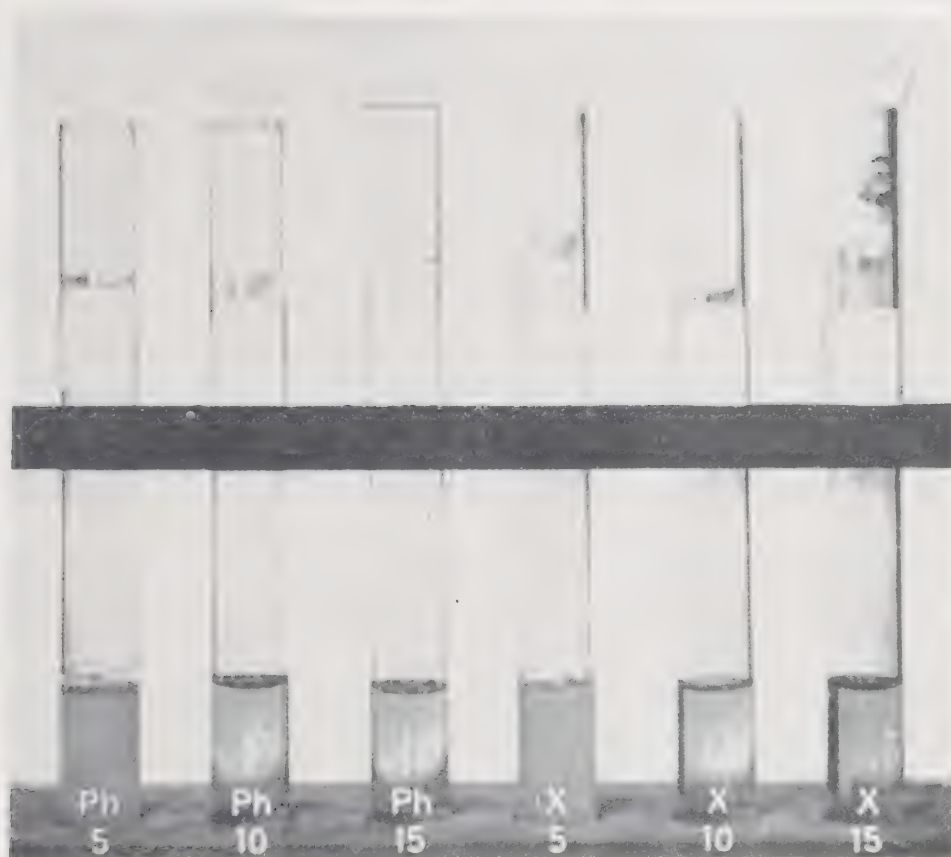


FIG. 13-5. Results of a phenol-coefficient test. A 1:100 dilution of phenol killed the test organism during 10- but not during 5-min. exposure, as indicated by the subcultures Ph, made after 5, 10, and 15 min. A 1:225 dilution of disinfectant X killed in the same time period, as indicated by subcultures X. Phenol coefficient of X is  $225/100 = 2.25$ .

basis to kill the cells in a defined volume of a 24-hr. culture of *S. typhosa*. Employ a different organism or different conditions, and the phenol coefficient for the test germicide could have a markedly different value. It has already been pointed out that the nature of the disinfectant and of the medium employed for subcultures may influence the results of such a test. Also, no consideration is made of the influence of the concentration coefficient  $n$  previously considered. This agent, if it has a lower value of  $n$  than phenol, will be more effective on equal dilution, and if a 20-min. test period were employed, its phenol coefficient might be of the order of 10 or 12.

This discussion points to the difficulties encountered in expressing on a numerical basis the relative merits of a group of agents having germicidal activity but possessed of widely diverse physical and chemical properties. It leads to the conclusion that the only safe test of an agent is



to determine how satisfactory it is under the conditions in which it is to be employed, bearing in mind the general principles involved in disinfection and borrowing when possible from the experience of others. A summary of such experience, compiled from the data and observations of numerous workers, will be presented at the end of this section.

**Miscellaneous Tests.** Additional methods of testing disinfectants and antiseptics are in use and have both merits and demerits as compared to a phenol-coefficient test. One method consists in soaking filter papers in a suspension of the test organism; after a given time the papers are removed and placed in appropriate dilutions of the disinfectant. After a suitable time the papers are transferred to broth and subcultures immediately made to reduce the bacteriostatic effect of any adhering disinfectant. Two or more agents can be compared, with each other and with an arbitrary standard such as phenol.

In another method the disinfectant is placed in a hole cut in, or a tube placed on, the surface of nutrient agar freshly inoculated with the test organism. The width of the area around the disinfectant in which no growth is evident after suitable incubation gives an indication of the bactericidal and bacteriostatic activity of the agent under these conditions. The test gives an idea of the relative strength of different agents and of their relative penetrating power in agar, but the results are rather difficult to compare with each other on a numerical basis. This method is also employed in determination of the concentration of penicillin and



FIG. 13-6. Cup-plate method for comparison of germicidal action of different agents

ether antiseptics. A similar method for the evaluation of the antiseptic properties of salves and similar water-insoluble vehicles is to note the width of the zone of inhibition of bacterial growth around a sample of the material placed on the inoculated agar. Other bacteriostatic tests have also been suggested, and in general they consist of variations in methods of determining the highest dilutions of an agent capable of restraining or preventing the growth of the test organism under particular conditions.

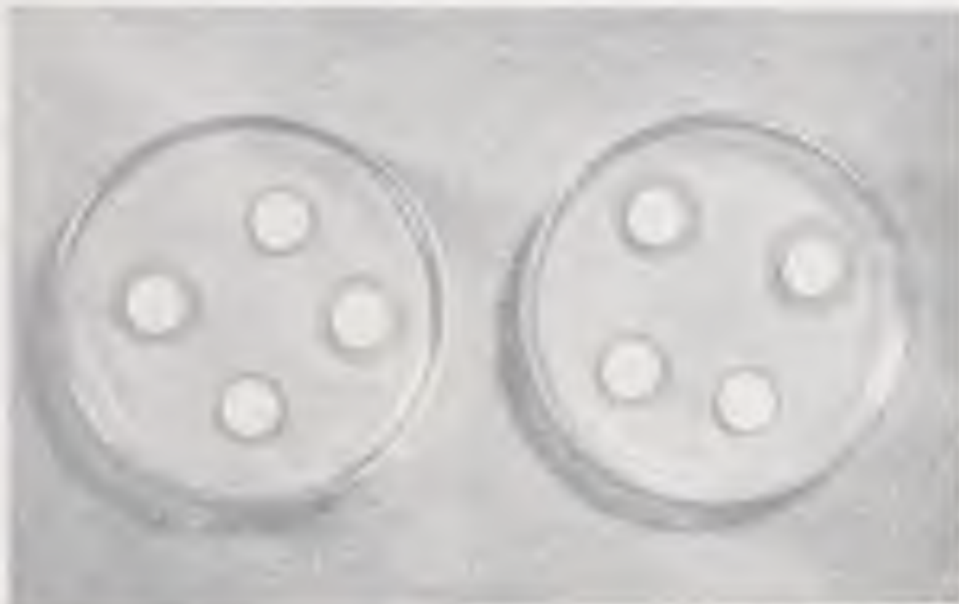


FIG. 13-7. Paper-disk method for the determination of the concentration of penicillin in a solution. The different sizes of the zones of inhibition of bacterial growth are due to differences in the amount of penicillin in the paper disks. The same type of a test is also employed for determining the sensitivity of a species to different antibiotics. (Courtesy of Eli Lilly and Co.)

Amongst the more recent tests proposed for the evaluation of antiseptics are ones proposed by Salle and by Bronfenbrenner. In the test proposed by Salle the efficiency of germicides is evaluated by testing them for their effect on the growth of living embryonic tissue and of bacteria. A toxicity index is determined on the basis of the ratio of the highest dilution of the disinfectant showing no growth of tissue to the highest dilution of disinfectant showing no growth of bacteria on subculturing each following 10 min. exposure at 37° C. to the disinfectant in the presence of a definite amount of organic matter. As an example, iodine in a 1/650 dilution was lethal to tissues and in a 1/3,520 dilution to staphylococci. Dividing 650 by 3,520 gives a toxicity index of 0.2 for iodine; in other words, approximately five times more iodine was re-

quired to kill the tissues than the bacteria. Phenol, with an index of 2.0, is twice as destructive to tissues as it is to bacteria, under the conditions of the test. With *Salmonella typhosa* as the test organism the toxicity index was 0.2 for iodine and 1.2 for phenol, indicating differences in toxicity of some agents against different organisms, but identical indices were obtained in other cases. Additional values will be given later. Toxicity indices suggest the relative efficiency of chemical agents intended for use as antiseptics, a low value indicating greater action against bacteria than against tissue cells. While it does not follow that an antiseptic with a low toxicity index will be harmless to tissues, the index does suggest a range of concentrations in which the agent would exert inhibitory power against the bacteria without causing too much damage to the tissues.

The test proposed by Bronfenbrenner is based on similar lines, with the exception that inhibition of oxygen consumption rather than of growth is employed as an indicator of lethal action. Still other tests involve the inhibition of phagocytosis, of development in the fertile egg, and of the development of infections in intracutaneous lesions in an animal host. All these tests aim at more efficient determinations of the possible efficacy of an agent to be employed in contact with living tissues for the control of invading or established microorganisms. They do provide additional data at least qualitatively, if not exactly quantitatively, significant in character.

The evaluation of chemotherapeutic agents requires even greater care, since they enter the circulating fluids of the body. It must be determined that the agents exert a bactericidal or at least bacteriostatic action, and the agents must be tested not only for general tissue toxicity but also for numerous pharmacological properties. Such tests run far afield from general bacteriology.

We have seen that no single, completely reliable method exists for the determination of the germicidal values of a variety of agents against different bacteria expressed with respect to any one standard substance. Of necessity, one must still determine the efficacy of an agent under the conditions in which it is to be employed. It is worth while to stress again that disinfectants in general are not miracle workers and that death of microorganisms is a gradual process, accelerated in rather definite relationships by increases in temperature and in concentration of the disinfectant, but often subject to the retarding influence of organic matter and to some extent of extraneous inorganic matter as well. A consideration of disinfectants and of disinfection in a general way might well conclude with a list of the factors desirable in an "ideal" disinfectant. Such a list could read as follows:

1. Active in low dilution
2. Low tissue toxicity, if employed as an antiseptic
3. Freedom from undue species specificity
4. Efficient in the presence of organic matter
5. Low coefficient of dilution
6. Low temperature coefficient
7. High power of penetration, i.e., low surface tension
8. Rapid in action, a high  $K$  value
9. Sufficiently soluble, stable, and homogenous
10. Chemical compatibility, e.g., lack of corrosive action
11. Low cost

A fortune awaits the individual who discovers an agent fulfilling these requirements!

### PRACTICAL METHODS FOR THE DESTRUCTION OF MICROORGANISMS

The development of methods for the destruction or control of pathogenic microorganisms or of organisms responsible for food spoilage has been one of the major contributions of microbiology to public health. It helped to bring about the development of modern surgical techniques; it helps in preventing the spread of contagious diseases; and it has made possible the preparation and preservation of perishable foodstuffs on a mass-distribution basis together with ensuring palatable and at the same time safe supplies of water and milk. The development of chemotherapeutic agents, particularly in the last decade, has made possible more efficient treatment of individual infections and in the long run more effective control of the spread of infectious agents. It is possible to consider here only some of the more prominent methods and materials employed for the control of the destructive action of microorganisms.

#### PHYSICAL AGENCIES

**Heat.** The agent most commonly employed for the destruction of microorganisms is heat. Fire itself destroys all forms of life, but it is hard to control and has limited application. In the laboratory it is employed for the sterilization of inoculating needles, but even here there are disadvantages. Rapid heating frequently causes material adherent on the needle to sputter, and organisms are discharged into the environment before they have been destroyed by the flame. Infections have resulted from this use of the free flame, and the technique must be carefully employed when working with pathogenic forms.

Dry heat is commonly employed for the sterilization of glassware in the laboratory. Death is a more gradual process in the absence of water in the environment of the cells, and experience has shown that it is neces-



sary to hold the materials to be sterilized at a temperature in the neighborhood of  $160^{\circ}\text{C}$ . for  $1\frac{1}{2}$  hr. to ensure sterility. This means that the material to be sterilized, not just the oven, must *reach* that temperature and be *maintained* at that temperature if sterilization is to be complete. The same holds true for any method in which heat is employed, since it is necessary for the heat to penetrate into every portion of the material to be sterilized, and many substances do exhibit considerable activity as



FIG. 13-8. Illustration of a constant-temperature incubator. Hot-air ovens for the dry sterilization of glassware are similar in general appearance but are more ruggedly constructed for operation at higher temperatures. (Courtesy of the Wilmot Castle Co.)



FIG. 13-9. Illustration of an autoclave for the sterilization of media or bandages. (Courtesy of the Wilmot Castle Co.)

insulators. The hot-air oven commonly employed for dry-heat sterilization is similar in construction to the oven used in the home for baking purposes.

Moist heat is employed more commonly than dry heat as a sterilizing agent because of the more rapid rate of death at a given temperature in the presence of moisture. It is not commonly employed for glassware because the sterilized material in this instance needs to be dry. Three types of application are in common use: boiling, exposure to a stream of steam, and steam under pressure greater than atmospheric with consequent increased temperature.

Boiling is a common procedure, particularly for sanitation, and at the boiling point of water vegetative cells of bacteria are killed within a

matter of seconds. Actually, if a suspension of bacterial cells is heated, all cells would be killed before the boiling point is reached. Spores are more resistant and may resist boiling for an hour or longer. To ensure sterility, it is therefore necessary to subject the material to be sterilized to a higher temperature. This is done by exposing the material to steam under pressure. At a pressure 15 lb. greater than atmospheric, a temperature of around  $120^{\circ}\text{C}$ . is developed, and under these conditions both spores and microorganisms are killed in 15 to 20 min., provided again that these biological agents themselves are at that temperature and are exposed directly to the steam. Sterilization under steam pressure is carried out in the laboratory in an autoclave, and a similar type of equipment, but on a much larger scale, is employed in the canning industry.

Flowing or live steam is not confined in a tightly closed container, and since its temperature is approximately  $100^{\circ}\text{C}$ ., death is a more gradual process than at the higher temperatures developed with steam under pressure. It is employed to reduce the danger of chemical breakdown of the material to be sterilized but is not particularly effective against many spores.

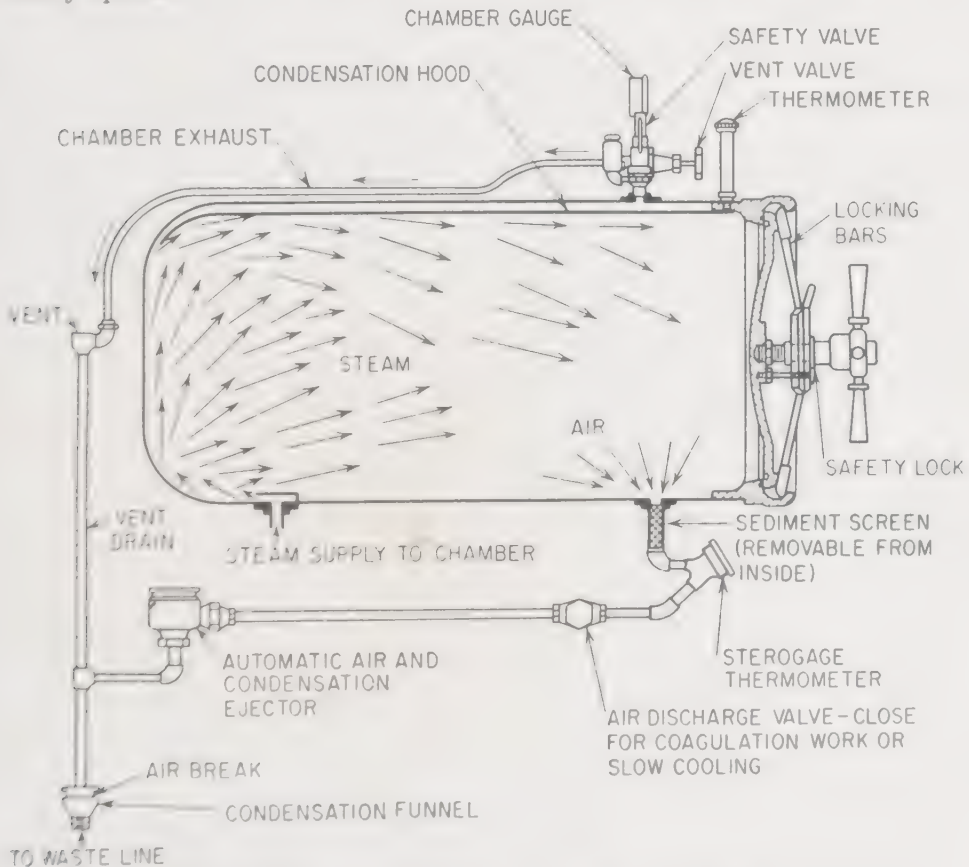


FIG. 13-10. Cross section of an autoclave. (Courtesy of the Wilmot Castle Co.)

Partial sterilization, or pasteurization, is carried out by heating the material at a temperature near  $60^{\circ}\text{C}$ . for 30 min., this time and temperature being sufficient to destroy the common pathogenic forms. Spores will not be destroyed in this procedure, and certain vegetative forms will likewise survive. The thermophilic bacteria, in fact, will thrive at this temperature.

Two indices of thermal resistance of bacteria and spores to killing have been used. These are the *thermal death point*, defined as the temperature at which the test organisms are killed in ten minutes, and the *thermal death time*, the time required for disinfection at a given temperature. Knowledge of thermal death times is of particular value in the canning industries. Withdrawal of heat, i.e., refrigeration, is not particularly destructive to most species of bacteria, but cold does inhibit the growth and activity of bacteria, and for that reason materials stored at low temperatures will not be decomposed rapidly by microbial action. Temperatures below freezing are more conducive to death of bacteria but some cells may survive.

**Light.** By light is meant that region of the spectrum of wavelengths or frequencies to which the human eye is sensitive (red to blue light) and the regions immediately adjacent thereto, the infrared and ultraviolet bands. Different portions of the light spectrum of frequencies have different effects on the bacteria, the

very short or ultraviolet rays being by far the most destructive. The destruction of bacteria by ultraviolet light is quite rapid, provided that all the bacteria can be exposed for a period of time to light of sufficient intensity. Since ultraviolet light is readily absorbed by ordinary glass, water, and many materials, it is absolutely essential to provide for direct contact of the bacteria with the light. Attempts to sterilize water or milk with ultraviolet rays are generally unsatisfactory on a large scale because of the absorption of light by these agents, and also because of the cost of a satisfactory apparatus and procedure. Numerous instal-



FIG. 13-11 An illustration of the germicidal activity of ultraviolet light. The agar in the petri dish was heavily inoculated and then exposed to the light from a mercury lamp, the central heart-shaped portion being blocked from the rays by a piece of paper.

tations of ultraviolet lights have been made in schools, offices, and public buildings in recent years in attempts to reduce the air-borne spread of

infectious agents. The value of such a procedure is still under test. There is no doubt that ultraviolet light in the region around 2,200 to 2,600 angstrom units is bactericidal, but the bacteria must come into contact with the light and absorb sufficient quanta of energy to ensure the death of the exposed cells. Death, elicited by this or any other agent, is controlled by the principles discussed earlier in the chapter. One drawback to the use of ultraviolet light is its action on the skin if exposure is continued over too long a period of time and to a much greater extent its action on the eyes. Even a short exposure of the eyes to ultraviolet light of moderate intensity is sufficient to evoke an inflammation known as conjunctivitis. The use of glasses or goggles which absorb ultraviolet light greatly reduces this danger.

**Osmotic Pressure.** High osmotic pressures are generally markedly inhibitory to most bacteria and in many instances do exert a slow but definite bactericidal action. The salting of meats, storage of foodstuffs in strong brines or sirups, and partial dehydration are methods employed for the production of high osmotic pressures and consequent reduction of spoilage brought about by microbial action.

**Filtration.** Some materials cannot be sterilized by heat or other agencies without undergoing marked alteration in their physical and chemical properties. Such materials in solution are frequently sterilized by filtration, a process in which microorganisms are removed by physical and chemical properties of the filter. Filters commonly employed in the laboratory for the sterilization of media or of serums, enzyme preparations, toxins, and other heat-labile (destroyed by heating) agents are constructed of clay, diatomaceous earth, plaster of paris, asbestos, sintered glass, or collodion.

On a large scale, filtration is most commonly employed for the purification of water supplies and for the removal of bacteria from treated sewage. Such filters, usually composed of layers of sand and gravel, do not completely sterilize water, but an efficient filter will remove the majority of the bacteria present. In addition to a physicochemical removal of the bacteria by the filter itself, other organisms, in particular, protozoa, aid in the removal of the bacteria by utilizing them as food. This will be considered further in the chapter on water bacteriology.

**Recapitulation of Physical Methods.** Summarizing practical applications of physical agents commonly employed for sterilization of various materials or for greatly reducing the numbers of bacteria, the following tabulation may be useful:

**Combustion:** Sterilization of inoculating needles. Destruction of contaminated, combustible material such as bandages and refuse

**Dry heat:** Sterilization of glassware

**Boiling:** Contaminated material in the home, in particular dishes and linens (some



tization). Syringes and needles. Surgical instruments, particularly those with a cutting edge.

Free steam. Laboratory media containing somewhat unstable components.

Steam under pressure. Culture media and solutions. Discarded cultures. Surgical instruments and supplies, such as bandages, gauze, gloves, and gowns. Also bandaging and other contaminated cloth. (A special autoclave with a vacuum attachment for drying the material sterilized is commonly employed.)

Heating below 100°C. Pasteurization of milk and fruit juices. Preservation of sera.

Ultraviolet light. Destruction of bacteria in air. Limited applications for special purposes.

Osmotic-pressure effects: Preservation of foodstuffs.

Filtration: Sterilization of media and special materials. Partial purification of water.

Removal of bacteria from suspensions of filtrable viruses.

## CHEMICAL AGENCIES

**Salts of Heavy Metals.** Mercuric chloride (bichloride of mercury, or corrosive sublimate) is a powerful germicide under favorable conditions. It is irritating to the skin, corrosive to metals, a strong poison if taken internally, and its action is greatly reduced by the presence of proteinaceous matter. Its use is primarily limited to scrubbing the hands and arms and to the disinfection of small glass and rubber articles, e.g., clinical thermometers and catheters. For this purpose a concentration of 1:500 or 1:1,000 is commonly employed. Mercuric iodide appears to have an activity similar to the chloride and is possibly somewhat less corrosive or irritating. Because of the efficacy of mercury ions as disinfectants, numerous organic compounds containing mercury (such as Merthiolate) have been developed in attempts to reduce the undesirable characteristics associated with mercuric chloride.

Various compounds of silver are germicidal, silver nitrate being commonly employed. It is effective in concentrations of 0.1 to 1.0 per cent applied on the skin and on mucous membranes, but it does have a cauterizing effect. Its toxicity index (Salle, p. 295) with staphylococci is 1.8, with typhoid bacilli 0.11. A few drops of 1.0 per cent (1:100) silver nitrate should be placed on the eyeballs of every newborn child to prevent the possible development of gonococcal infection of the conjunctiva. (Penicillin is also effective for this purpose.) Any irritating effect can be prevented to a considerable extent by subsequent washing of the silver salt from the eyes with the aid of physiological saline. Silver is sometimes incorporated into organic compounds to reduce its cauterizing action. In combination with proteins, in preparations such as Argyrol, the germicidal activity remains fairly high, and the toxicity indices are similar to those for silver nitrate itself.

Finely dispersed or colloidal metallic silver has been suggested for use

as an antiseptic or disinfectant, owing to the oligodynamic action of heavy metals. This term suggests the toxic effect of extremely minute amounts of heavy metals on living organisms. Silver does exert a marked bactericidal or oligodynamic action which can be readily demonstrated (see Fig. 13-12) in the laboratory by placing a silver coin in the middle of agar in a pour plate. Growth of bacteria such as *E. coli* will be prevented in a fairly large area around the coin, and around this area of inhibition or death of bacteria can be noted a ring of stimulated growth (Hueppe's law). Silver utensils have a bactericidal action as evidenced by studies of Burrows on the possible transmission of bacteria by use of a common communion cup made of silver. A similar but weaker effect is noted with copper. Copper salts are not as bactericidal as silver salts, but they are lethal to many species of algae in a concentration of one part in many million parts of water. Copper sulfate is frequently employed to control the growth of algae in water supplies. Sprays containing copper salts, such as Bordeaux mixture, are widely employed for the control of fungus infections in orchards.

Other heavy metals or their salts are not employed to a great extent as bactericidal agents, although salts of bismuth and of arsenic have high activity against certain spirochetes and trypanosomes and, together with mercury compounds, are used in the treatment of infections produced by these microorganisms.

**Halogens.** Chlorine is used extensively as a disinfectant for the treatment of drinking-water supplies, sewage, and water in swimming pools. It is also used to some extent in water for washing dishes in public places and for a variety of sanitary procedures. For large-scale use chlorine is commonly employed in liquid form; for smaller operations compounds liberating chlorine are more readily available. The mechanism of the bactericidal action of chlorine appears to be primarily one of oxidation, although direct chlorination of amino groups in proteins may also play a part. Chlorine reacts with water to give hydrochloric and hypochlorous acids, the latter compound acting as a strong oxidizing agent, according to the following reactions:

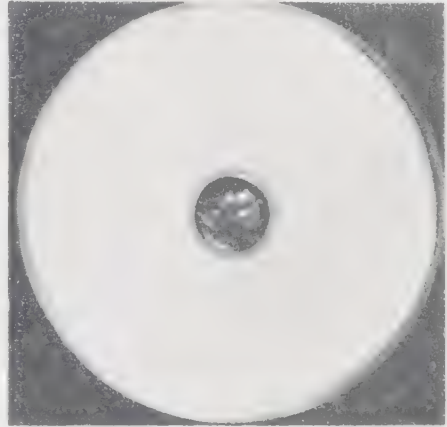
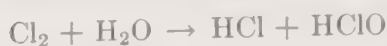
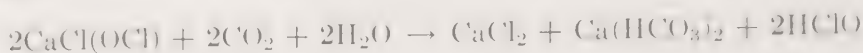


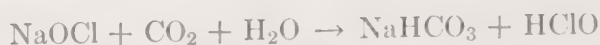
FIG. 13-12. An illustration of the oligodynamic action of silver. Note the absence of growth in the area immediately surrounding the dime in the pour plate of *Escherichia coli*.



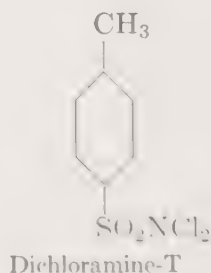
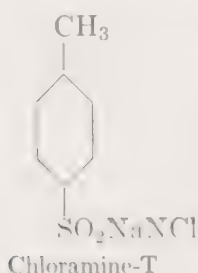
Calcium chlorohypochlorite (bleaching powder) or sodium hypochlorite, the most common substitutes for liquid chlorine, react in a manner similar to chlorine, generating hypochlorous acid in reactions which can be represented as



and



Compounds (chloramines) containing active chlorine attached to a nitrogen atom in  $-\text{NH}_2$  or  $-\text{NH}$  groups, one or more hydrogen atoms of which have been replaced with chlorine, also react in a similar manner. Ammonia is frequently added to water to be treated with chlorine, reacting with the latter to yield chloramines. Chloramines are decomposed with the formation of hypochlorous acid at a slower rate than when chlorine or salts of hypochlorous acid are employed alone, and although the bactericidal action is decreased to some extent, the activity is extended over a longer period of time. Chloramines in general are somewhat less irritating to the eyes than chlorine itself, and for this reason chlorine plus ammonia is frequently employed in the treatment of swimming-pool water. Similar substitution compounds of ammonia, in which one of the hydrogen atoms is replaced by an organic molecule, e.g., chloramine-T and dichloramine-T,



are frequently employed as antiseptics in the treatment of infected wounds. Their toxicity indices lie below 0.5.

Chlorine is effective in extremely small amounts, not more than 0.5 to 1.0 part per million parts of water being sufficient to destroy all the common bacterial pathogens in a water supply if the organic content of the water is not high and if there is a residual chlorine concentration of 0.1 parts per million 20 minutes after chlorination. The protozoan agent, *Exdamoeba histolytica*, of amoebic dysentery and many viruses

are more resistant to chlorine than are the bacteria, and such agents of infectious disease may survive in chlorinated water supplies.

The sodium and calcium salts of hypochlorous acid are as effective as chlorine, but they do tend to break down spontaneously on storage, and their effective chlorine content is thereby reduced. Care, therefore, must be employed to use sufficient material to obtain an effective chlorine concentration.

Tincture of iodine, a 2.0 per cent solution of iodine in an alcoholic solution, is commonly employed as a household antiseptic for the treatment of minor wounds and in the surgery for the attempted disinfection of the site of an operation. The in vitro tests of Salle and coworkers indicate a very low toxicity index, 0.2, with both staphylococci and typhoid bacteria as the test organisms. Tincture of iodine is a handy agent for use on camping trips, both for use as an antiseptic and in the treatment of suspected drinking water. It has been found that two drops of tincture of iodine per quart of water will render most doubtful waters safe for drinking purposes in  $\frac{1}{2}$  hr.

**Acids and Alkalies.** The bactericidal action of most acids depends to a considerable extent upon the concentration of hydrogen ions produced therefrom. Since a high concentration of acid is required to develop a low pH, the use of acids as disinfectants is greatly limited. Boric acid is used to some extent as an antiseptic in eye washes, but its bactericidal and bacteriostatic properties are extremely limited.

The strong alkalies such as potassium hydroxide are bactericidal against most species of bacteria, *Mycobacterium tuberculosis* being an important exception. Fresh unslaked lime ( $\text{CaO} + \text{H}_2\text{O} \rightarrow \text{Ca(OH)}_2$ ) is also fairly effective and is frequently employed in the disinfection of excreta. The use of either acids or alkalies is limited by their destructive action on many materials.

**Organic Compounds.** Phenol (carbolic acid) is one of the oldest commonly employed disinfectants. Its use is rather limited at the present time although it is fairly effective in the disinfection of excreta, if employed in 5 per cent concentration. It has been replaced to a considerable extent by cresols, which are more effective disinfectants but which, as a result of their low solubility in water, must be employed as emulsions. Lysol, a trade name for a linseed-soap emulsion of cresols in water, has a phenol coefficient of 5. Both phenol and the cresols have toxicity indices in the neighborhood of 2.0. Lysol, in 2 per cent solution, is commonly employed in the laboratory as a disinfectant for washing the hands and for pipettes, slides, and similar contaminated glassware before the latter are placed in the autoclave for sterilization. Emulsions of orthohydroxydiphenyl (O-syl) also have a high phenol coefficient and less pronounced odor, and may be used in place of Lysol.



Ethyl (grain) alcohol is used to a limited extent as a disinfectant, particularly in swabbing the site of an injection or operation and in containers for clinical thermometers. Its bactericidal effect is generally stated to be most pronounced in 60 to 70 per cent concentration in water, too little water markedly reducing its efficiency. Recent studies suggest that 95 per cent alcohol may be most effective. The toxicity of alcohols against bacteria increases with their molecular weight. Ether has some germicidal action, being more inhibitory to bacteria than viruses. This renders it of some value as an agent for use in the purification of viruses, since it can be employed for the extraction of lipoidal material and at the same time it destroys bacterial contaminants.

Certain organic mercurials—Mercurochrome, Metaphen, and Merthiolate—have value as antiseptics or preservatives for biologicals such as serums and vaccines. The disinfectant power of Mercurochrome appears to be greatly overrated, while Merthiolate and Metaphen seem to be rather effective agents. The latter two are employed for the disinfection of skin and of implements and as antiseptics in minor injuries. They appear to have greater bacteriostatic properties than many other common antiseptics and for this reason difficulties are encountered in evaluating their actual worth as disinfectants. They are less irritating than inorganic mercury compounds such as mercuric chloride. Against *Salmonella typhosa* the toxicity indices (Salle) are 0.4 and 1.6 for Metaphen and Merthiolate, respectively. The corresponding values with *Micrococcus pyogenes* var. *aureus* as the test bacterium are 1.5 and greater than 169.

Soaps in general appear to be fairly effective germicidal agents, and in addition their cleansing properties aid in the mechanical removal of bacteria. Soaps, like the synthetic detergents, can be classified as surface-active agents. If a substance becomes oriented and concentrated at the boundary or interface between two systems, e.g., between a layer of oil and one of water, it brings them into more intimate contact. Such substances are known as surface-active agents and they promote wetting and penetration. If the substance promotes and stabilizes the dispersal of one substance in another, it is called an emulsifier. Soaps and detergents may act both as surface-tension-reducing agents and as emulsifiers. Some medicated soaps appear to be more effective than ordinary soaps as disinfectants.

Many of the synthetic detergents are anionic compounds, ionizing in water to give negatively charged organic ions such as  $\text{RSO}_3^-$ , a substituted sulfonic acid. The cationic detergents generally are quaternary ammonium salts,  $\text{R}_4\text{NCl}$  where the R groups are the same or different organic radicals. Their ions are positively charged and are capable of

neutralizing the action of the negatively charged soap and detergent ions. A number of the cationic agents appear to possess marked germicidal activity even in high dilutions. They are readily adsorbed by negatively charged bacteria, and the end result appears to be a deposition of a layer of the wetting agent over the surface of the cell. This eventually leads to death of the cell, but there is evidence that actual death in many instances occurs at a very slow rate. When employed under conditions in which the adsorbed layer may later be set free, e.g., by soaps, their germicidal value is greatly reduced. This is another illustration that the criteria for distinguishing between living and dead bacteria are by no means satisfactory, and suggests that these agents may be bacteriostatic rather than bactericidal in action in high dilution.

The wetting agents, like soaps, greatly reduce the surface tension of aqueous solutions, and this enables the agents to penetrate into cracks and crevices relatively inaccessible to ordinary solutions. This power of penetration increases their value as antiseptics and disinfectants. Soap, or detergents, and hot water remain of great value in sanitization around the home, in the kitchen, the laundry, and the bath.

Many dye-stuffs are relatively strong germicidal agents, although they tend to be rather selective in their action (see Fig. 13-13). Gentian (crystal) violet is commonly employed as an antiseptic or bacteriostatic agent against the gram-positive cocci. Acriflavine and proflavine are two dyes claimed to be more effective antiseptics than gentian violet, and they have been employed to a limited extent in local chemotherapy.

Formalin (a solution of formaldehyde in water) is a fairly effective germicide, but its odor and irritating characteristics markedly limit its use. Formaldehyde gas is employed to some extent as a fumigant, but for it to be effective, the humidity of the air must be relatively high, again indicating the importance of water in disinfection. The aerosols, exceedingly fine droplets of materials such as propylene glycol which exert bactericidal action when suspended in the air, may have some value as fumigants but appear to be most effective as bactericidal agents for use in the control of air-borne infectious agents.

### CHEMOTHERAPEUTIC AGENTS

There is no exact definition of chemotherapy, and use of the term here will be confined to the employment of chemical agents produced outside the animal body and generally introduced into the circulating fluids of animals in an attempt to inhibit or destroy the causative agent of a specific disease. It is not therapy in the broad sense of the term in that the agent does not necessarily have any healing properties. Attention



FIG. 13-13. Contact prints illustrating the selective action of gentian violet on gram-positive bacteria. Growth of a gram-positive species (lower half of the plates) was completely inhibited by 1:1,000,000 but not by 1:5,000,000 gentian violet, while a gram-negative species grew in the presence of both concentrations of the dye.

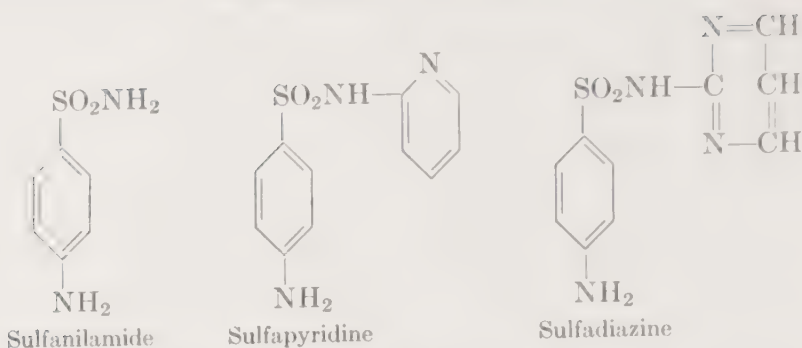
is focused primarily on the relationship between the parasite and the drug, the host being considered for the most part only in regard to the toxicity of the drug.

In early medicine, herbs were the main source of therapeutic material, definite chemical compounds being introduced in relatively recent times. One of the earliest specifics was mercury or mercury compounds, which were introduced for the treatment of syphilis in the sixteenth century. Quinine as a specific for malaria was introduced into Europe in the seventeenth century. In both cases the microbial origin of the diseases was not recognized, with the result that in neither was therapy on a rational basis.

With the establishment of the germ theory of disease, as a result of the studies of Pasteur and of Koch, interest began to develop in the possibility of the destruction, with the aid of specific chemicals, of the causative microorganism within the body. Paul Ehrlich reasoned that there are specific groups or "receptors" on the cell, and if these groups could be blocked or inhibited, the resultant damage would lead to the death of the cell. If a pathogenic bacterium possessed a group entirely different from any on the cells of the host, then the possibility existed that a compound could be found which would react specifically with that group. This would lead to inhibition or death of the parasite with little or no damage to its host. The hope of chemotherapy by competitive inhibition of enzymes is that a similar enzyme is not present in, or at least is not essential for, the normal functions of the host.

An arsenic-containing compound, atoxyl, was shown by Thomas in 1905 to be of value in the treatment of trypanosome infections of mice. This led Ehrlich to test the possibilities of organic arsenicals as chemotherapeutic agents against syphilis, and he synthesized and tested many new compounds. The 606th compound to be tested (1909) was salvarsan, and it was found to be fairly effective in the treatment of the disease. Thousands of other compounds were tested in various laboratories to determine their possible value as "magic bullets," but only a few of these were found to have any value as internal "disinfectants." The problem appeared hopeless until the discovery of the chemotherapeutic properties of the dye prontosil by Domagk in 1935. It was soon found that prontosil broke down in the animal body with the formation of *p*-aminobenzenesulfonamide (sulfanilamide) and that this compound was the effective agent in the treatment of streptococcal and other infections. Attempts were made at once to alter the structure of the sulfanilamide molecule in the hope of obtaining more effective chemotherapeutics. This led to the discovery of sulfapyridine, sulfadiazine, and other sulfa drugs, all chemical derivatives of sulfanilamide.





**The Sulfa Drugs.** The sulfa drugs vary somewhat in their action against different bacteria and in their toxic properties towards the animal body. In general they have proved to be most effective against infections produced by the gram-positive and gram-intermediate cocci, e.g., staphylococci, streptococci, pneumococci, and gonococci. Unfortunately these organisms, and in particular the gonococci, tend to become resistant to the sulfonamides on exposure to sublethal amounts of the drugs, and the drug-fast strains so produced no longer respond to amounts of the drug that can be tolerated by the host.

The sulfonamides are primarily bacteriostatic rather than bactericidal in their action, and Woods demonstrated that *p*-aminobenzoic acid (PAB), which was later shown to be an essential substance or "essential metabolite" for many different types of cells including mammalian, virtually abolished the bacteriostatic action of sulfanilamide. The action of the sulfa drugs is believed by many workers to be the result of interference with the function of PAB in the cell, in other words, competitive inhibition of an essential enzyme system or reaction (the jamming of a lock by a key which does not fit perfectly). This is supposed to be due to the structural similarity between the metabolite and the drug, a similarity apparent from the following structural formulas:



The entire story is not known as yet, but the speculations and work on the problem may lead to the development of more and better chemotherapeutic agents. It appears that the major effect of the sulfa drugs is to

inhibit the synthesis of a particular structure (pteroyl) needed by the bacteria and other forms of life. These pteroyl compounds are synthesized with *p*-aminobenzoic acid as one of the intermediate compounds, and they are related to the growth factor folic acid. Bacteria requiring pteroyl compounds or folic acid preformed in the culture medium are relatively insusceptible to the sulfa drugs. For further reading in this interesting field the student should refer to the recent reviews by Dubos, McIlwain, Lounie, Hotchkiss, and others and to a historical treatment by Galdston.

**Antibiotics.** A two-sided antagonism exists in an infectious disease, the invading parasite exerting a destructive action on the host and the host in turn attempting to neutralize the deleterious effects of the parasite. Specific chemical agents may be liberated by the parasite to aid in its attack on the host. Likewise a similar situation may exist in nature when one organism tries to gain an ascendancy over another in its environment. This suggests a possible purposiveness on the part of a micro-organism which may not actually exist. At least it is known that some organisms do produce substances inhibitory to the growth of others.

Pasteur observed as early as 1877 that the infectivity of the anthrax bacillus was at times reduced by the presence of a second bacterium. This suggested to his inquiring mind the possibility that antagonistic action of this sort might eventually be employed in the treatment of infectious diseases. Around the beginning of this century it was observed that *Pseudomonas aeruginosa* (*B. pyocyaneus*) produced a product, termed pyocyanase, which was inhibitory to the growth of a number of species of bacteria. This material appeared to have chemotherapeutic possibilities, but its use was found to be limited by its toxicity to man. Other antibiotics were demonstrated in ensuing years, but the possibility of their use in chemotherapy appeared remote until the discovery in 1939 of tyrothricin by Dubos. This material was found to be composed of two fractions, gramicidin and tyrocidin, the latter possessing local chemotherapeutic properties but being too toxic for general internal use.

Fleming's discovery of penicillin in 1929 came as the result of his observation that an accidental mold contaminant on an agar plate culture of staphylococci inhibited and finally destroyed the staphylococci in its vicinity. The antibiotic or antagonistic activity of this fungus, *Penicillium notatum*, was traced to the elaboration by the mold of a soluble material which Fleming called penicillin. The addition of this material in crude form to culture media suppressed the growth of gram-positive and allied forms but had little effect on the true gram-negative bacteria. Limited tests suggested that penicillin might have possibilities as a "magic bullet."

With the development of the sulfa drugs and the increased need in

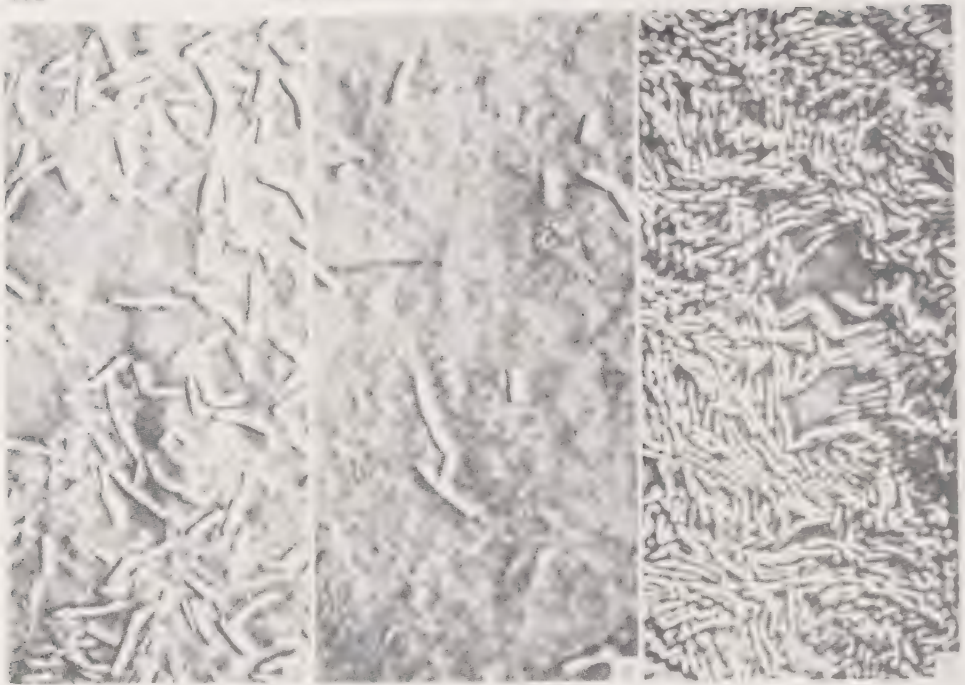


FIG. 13-14. Influence of streptomycin on the growth of *Bacillus subtilis*. Part (A) represents the inoculum of *B. subtilis* on agar adjacent to a colony of *Streptomyces griseus*; (B) is the same field two days later, showing evidence of the lytic activity of streptomycin; and (C) shows growth away from the antibiotic. (Photomicrographs by A. C. Lonert, courtesy of General Biological Supply House, Inc., Chicago.)

wartime for bactericidal agents to prevent or counter bacterial infections of wounds, attention was again turned to penicillin in 1939 by a group at Oxford under the leadership of Florey. Their tests produced evidence of its possible value as a chemotherapeutic agent, and in the following years its value has been amply demonstrated. (The mass production of penicillin is a miracle of modern microbiology and will be considered under industrial applications.)

Numerous other antibiotic agents have been discovered, and the search is still in progress. Most of these agents are too toxic to man to be of value as chemotherapeutics. At the present time streptomycin (or dihydrostreptomycin), elaborated by *Streptomyces griseus*, appears to be the most promising, after penicillin. The tetracyclines—tetracycline (Achromycin or Tetracycl), chlortetracycline (Aureomycin), and oxytetracycline (Terramycin)—are of considerable value and tend to have a wider range of antibacterial activity than penicillin. Chloramphenicol (Chloromycetin, which can be prepared synthetically), bacitracin, neomycin, polymyxin B and E, and erythromycin are other antibiotics of value in the treatment of various bacterial infections. One trouble encountered in the clinical use of an antibiotic is the development of



resistant mutants. These variants, however, are generally susceptible to other antibiotics. At times, mixtures of antibiotics are employed in chemotherapy or, in the case of tuberculosis, an antibiotic—streptomycin—may be employed in conjunction with a relatively simple antitubercular drug, isoniazid. A few agents have been isolated that may serve in the treatment of fungal infections, but more work is required before their value is established. It is remarkable that these agents have been found and their value in therapy established during a very short period of time. The antibiotic industry has grown by leaps and bounds, in about ten years, to the point where in 1954 more than 2,000 tons of antibiotics were produced in the United States; these products have a market value of over \$250,000,000. It is of biological interest that many of the antibiotics are most active against gram-positive bacteria, indicating a fundamental difference between the gram-positive and the gram-negative forms.

In the treatment of an infectious disease it is important that the causative agent be isolated and its sensitivity towards antibiotics be determined by laboratory tests. Paper disks (see Fig. 13-7) are available commercially for these tests. Staphylococci, for example, were generally sus-

TABLE 13-1. SOME COMMON DISEASES AND ANTIBIOTICS OF CHEMOTHERAPEUTIC VALUE AGAINST THEIR CAUSATIVE AGENT

Infection	Causative agent	Penicillin	Streptomycin	Chloramphenicol	Chlor-tetracycline
Gonorrhea.....	<i>Neisseria gonorrhoeae</i>	+	+	+	+
Pneumonia.....	<i>Diplococcus pneumoniae</i>	+	+	+	+
Lymphogranuloma venereum.....	a virus(?)	—	—	+	+
Scarlet fever.....	<i>Streptococcus pyogenes</i>	+	—	?	+
Staphylococcal infections.....	<i>Micrococcus pyogenes</i>	+	?	?	+
Syphilis.....	<i>Treponema pallida</i>	+	+	+	+
Tuberculosis.....	<i>Mycobacterium tuberculosis</i>	—	±	?	?
Typhoid fever.....	<i>Salmonella typhosa</i>	—	?	+	+
Typhus fever.....	<i>Rickettsia prowazekii</i>	—	—	+	+
Viral pneumonia....	a virus	—	—	+	+
Whooping cough....	<i>Hemophilus pertussis</i>	—	+	+	+

+ effective

— ineffective

? of doubtful therapeutic value



ceptible to penicillin, but many penicillin-resistant strains have developed or may arise during the course of penicillin treatment. This can be indicated by sensitivity tests, which also enable the physician to select the antibiotic most active against the causative agent.

**Chemotherapy and Demography.** Lourie has pointed out that the advances in chemotherapy and in sanitation in recent years have led to the potential control of human infectious diseases on a mass scale. These advances in turn are reflected in better control of diseases of lower animals and to a lesser extent of those of plants. Lowering of the death rate of the human population by control of infectious diseases increases the longevity of man and in turn the population to be supported in a given area. Chemotherapy and modern sanitation may well play an increasingly large part not only in the relief of suffering but also in the aggravation of some of mankind's most serious problems, the supplying of the basic needs of man. Solving some of our problems, it creates new ones. Advances must keep apace in the treatment or prevention of diseases of plants and animals necessary to fulfill the nutritional requirements of their parasite, man.

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## CHAPTER 14

### MICROBIAL VARIATIONS

Microorganisms, like all other forms of life, are subject to change. In the early days of bacteriology, one school of thought postulated that a rapid and almost unlimited variation could occur in a single species. At times it predominated in the form of a rod, as a coccus, or as a curved form. There is little doubt at the present time that many of these "variations" were due to mixed cultures or to contamination of supposedly pure cultures, but many of the early investigators were strongly convinced of the pleomorphic character of bacteria. This concept of pleomorphism was defended by von Nägeli, who suggested in 1877 that all bacteria belonged to one species, which could exist in a variety of forms. The development of pure-culture techniques by Koch made more accurate studies of bacteria possible and in time led to the development of the Cohn-Koch concept of monomorphism or the fixity of species. The concept that a given species maintains its physiological and morphological characteristics with great constancy dominated the field of microbiology for many years, even in spite of observations such as those of Pasteur, who had shown that *Bacillus anthracis* decreases in virulence and loses its power of sporulation when cultivated at 42°C.

In old cultures or in cultures developing under abnormal conditions, many workers observed *involution forms*, cells markedly different in appearance from the forms observed in cultures 24 to 48 hr. old, and the doctrine of monomorphism in time had to be limited to organisms cultivated under a given set of conditions, it being admitted that a few cells might undergo nontransmissible changes in size or form. Nevertheless, differences in morphology, pigmentation, colonial form, virulence, and biochemical properties were frequently observed even under carefully controlled conditions, and by 1915 the doctrine of fixity of species became untenable. It is now recognized that a bacterial species, isolated in pure culture from a single cell, may undergo variation, transient or permanent, but that these variations are subject to natural laws. Transient variations occur with age of the culture or with the nature of the environment as two important controlling factors. More permanent, transmissible variations appear to occur spontaneously and to be genetically controlled.

although our knowledge of the genetics of bacteria is primarily by analogy with that of higher forms. However, transmissible variations may also be induced artificially.

**Variations of Bacteria during Growth.** Numerous experimental observations have definitely established that variations occur which are definitely linked with the age of the culture. Quite frequently it is stated that these variations occur with age of the cells, but it is difficult to establish the actual age of a cell. Let us consider two cells which have just been formed as a result of binary fission of the parent cell. Are these cells infants which may mature in a matter of minutes and within 20 to 30 min. give rise to a total of four new infants? Later when there are 1 million cells in the culture derived from the original cell, are the 2 million cells at the moment of fission of their parent cells of the same age as the two cells first considered? Or do bacterial cells age only after they cease multiplying in the culture?

The cells of early generations of many bacterial species cultivated under favorable conditions are markedly different from those of generations produced as the population nears its maximum. The mean cell volume increases during the lag period of growth and reaches a maximum near or early in the logarithmic period of growth. Cell volume then decreases as logarithmic growth continues and reaches a minimum value by the time the growth rate markedly decreases. Along with changes in cell size may also be noted marked and generally parallel changes in metabolic activity per cell (see Fig. 12-5), whether one measures food or oxygen consumption or the production of metabolic wastes such as carbon dioxide, ammonia, or heat. Cells from young cultures exhibit quite uniform staining properties and a more marked affinity for basic dyes, the latter along with reduced agglutinability by acids and a lessened electro-negative charge, suggesting that the over-all isoelectric point is more on the acid side than that of cells from older cultures. The earlier generations are capable of adapting themselves to a change in their diet more readily than older generations, but on the other hand these same cells have less resistance to inimical agents such as salts, phenol, dyes, or marked temperature changes. Sherman and Albus, Henrici, and others have developed the concept that cells of these early generations are "physiologically young" and that the properties observed during the early part of the growth curve are to a great extent the result of the embryonic nature of the bacteria at this time.

With increasing age of the culture there is a continuous decrease in the amount of available energy and of essential building material per cell and at the same time an increase in the concentration of waste products in the environment. Such factors undoubtedly influence the behavior and activity of the cell. Physiological youth in bacteria may

in part be a response to the environment rather than being due to actual age of the cells alone. The environment is continuously changing as the bacteria grow in a culture, and it could be that the cells truly age only after they cease to multiply.

In old cultures there is no doubt that cells may alter in their morphology, and in other properties as well, with increasing age of the cell, but at the same time there are less pronounced changes occurring in the nature of their environment. Involution forms become readily apparent with many species of bacteria, some such as *Corynebacterium diphtheriae* producing club-shaped cells many times larger than the average cell, others such as species of *Rhizobium* assuming the form of stars, crosses, and bizarre forms of various sorts. At one time it was strongly believed that the rhizobia went through a definite life cycle. Banded rods observed in older cultures, or in the root nodules on leguminous plants, were believed capable of liberating small, nonmotile cocci which could increase in size, develop flagella, and assume an ellipsoidal form. This latter form was said to elongate and become vacuolated, the chromatin dividing into a number of bands which in time escaped in the coccoid form from the rod and began the cycle anew. More recent studies raise doubt as to the existence of such a life cycle, the banded condition being caused by the deposition of fat in the cells, which restricts and compresses the cytoplasm. The small coccoid and oval cells are produced by fission during periods of restricted growth and hence are to be considered as a response to aging and environment rather than as any particular stage in the development of the culture. Similar pathological changes may be observed in other forms of life. This problem, however, needs further study before a definite conclusion is established.

**Specific Adaptive Variation.** When an organism such as *Listeria monocytogenes* is cultivated on a blood-agar slant, it gives rise to the development of relatively small and fairly uniform cells, flagellated only in the early logarithmic period of growth. On cultivation in the presence of glucose under partial conditions of anaerobiosis, this organism shows marked variation in morphology, existing as long rods and exhibiting many curved forms (see Fig. 14-1). On transfer back to blood agar, the "normal" form of the species is again observed. Here we may observe marked variation in flagellation with age of the culture, in morphology with variation of the culture medium. *Escherichia coli*, grown on Sabouraud agar, is quite filamentous in appearance (see Fig. 14-2) as compared with cells from ordinary agar.

Many bacteria, as we have seen, are capable of multiplying under a wide range of conditions, tolerating marked changes in either the physical or chemical nature of their environment. In some instances, little or no change in the biochemical or morphological properties of the cells





FIG. 14-1. Variation in morphology of *Listeria monocytogenes* from blood agar (A) compared with cells grown under semianaerobic conditions in a dilute agar-glucose broth (B).

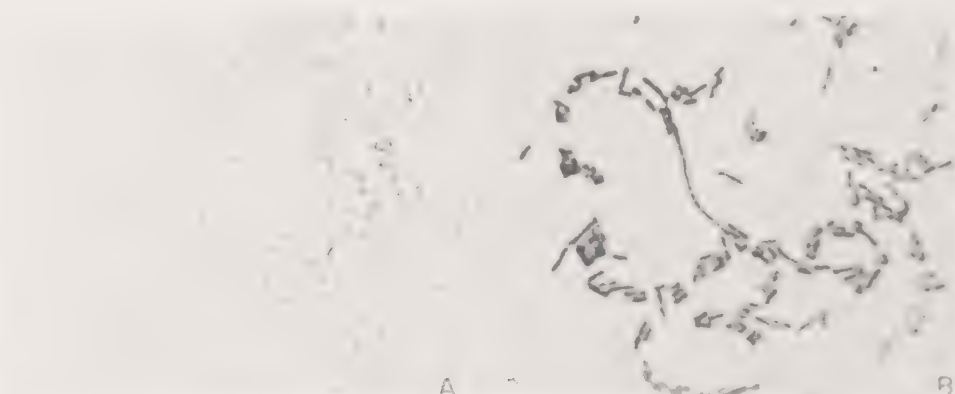


FIG. 14-2. Variation in morphology of *Escherichia coli* grown on plain agar (A) and on Sabouraud agar (B), pH 5.6.

is observed; in other instances, such as the case cited above, marked changes may be observed. The elongated form of rod-shaped bacteria is probably due to an internal force disposed along the long axis of the cell, which opposes the rounding influence of surface tension. When the surface tension of a medium is lowered by the addition of a surface-tension depressant such as sodium ricinoleate, an organism such as *Escherichia coli* in young cultures may exhibit very long, filamentous forms. The addition of calcium chloride to the medium increases surface tension to a slight extent, and the cells become shorter and more oval in appearance than when cultivated in ordinary broth. Inorganic ions exert a wide variety of effects, the concentration of iron in the medium markedly influencing toxin production by *Corynebacterium diphtheriae*. Other ions at times influence capsule formation, sporulation, type of

colony produced, etc. Increased salt concentration, or the addition of phenol to the medium, inhibits or prevents the formation of flagella by *Proteus* species. The loss of flagella results in the production of a more discrete colony, the O form, and a difference in the antigenic structure of the cells as compared with the normally flagellated cells, which give rise to spreading, or H, colonies. Certain of the effects produced by alterations of the environment may be specific, others nonspecific in character.

The enzymic constitution of bacteria may undergo wide variation in relative amounts or in actual constituent enzymes with change in the environment. *Azotobacter* is able to grow in the presence of ammonia when molybdenum and vanadium are absent from the culture medium, but this genus is unable to fix atmospheric nitrogen and grow in a medium devoid of nitrogenous compounds in the absence of the above inorganic elements. The addition of one of these elements to the medium enables the organism to fix nitrogen and to grow. Other inorganic elements affect the production or activity of other enzymes, magnesium being essential for the zymase complex, calcium for the production of gelatinase, iron for the production of the  $\alpha$  toxin of *Clostridium perfringens*, oxygen for pigment production by *Serratia marcescens*, and the production of certain toxins by staphylococci is markedly affected by both the oxygen and the carbon dioxide tension of the environment.

The ability of *Penicillium notatum* to produce penicillin is markedly influenced by the nature of the medium, penicillin production on a large scale becoming economically feasible when it was observed that the substitution of lactose for sucrose and the addition of corn steep liquor to the culture medium markedly increased penicillin formation. At the same time it must be pointed out that individual strains vary markedly in their ability to produce penicillin in a given medium, thus suggesting that in nature a species may be continuously undergoing change.

Certain enzymes are always present in a given species although their relative amounts or activity may vary. Other enzymes appear only as a result of the addition of a different foodstuff to the culture medium. There are, of course, limits to the range of enzymes which a given species may produce, and the resultant metabolic behavior is utilized in the classification of bacteria, but unfortunately these limits are not always definitely known, and many of the now-recognized species may be variant strains of one species. Two brothers adapted to entirely different diets may excrete somewhat different waste products, but they are still of the same species!

The normally occurring enzymes are commonly known as *constitutive enzymes*, the group appearing as the result of a response to the environment are known as *adaptive enzymes*. Karstrom, in 1930, clearly demonstrated that a lactic acid bacterium, *Leuconostoc mesenteroides*, in

washed suspensions was always capable of fermenting glucose regardless of the presence or absence of glucose in the original culture medium. Washed suspensions of the same organism were unable to ferment lactose unless lactose had been present in the original culture medium. When the organism was cultivated in the presence of lactose, it readily fermented lactose, and glucose as well. But when the lactose-fermenting cultures were passed through lactose-free media again, they lost their power to ferment lactose. Karstrom concluded that the enzymes involved in the fermentation of glucose are always present in the strain of bacteria he studied; hence they are constitutive enzymes, while the lactose-fermenting enzymes, which appear only as a response to the presence of lactose in the culture medium, are adaptive enzymes. From his studies, summarized in Table 14-1, it is apparent that the enzymes involved in the fermentation

TABLE 14-1. ADAPTION OF *Leuconostoc mesenteroides* TO THE FERMENTATION OF VARIOUS SUGARS \*

Original medium, broth plus sugar	Sugars fermented by washed suspensions					
	Glucose, fructose, or mannose	Galac- tose	Arab- inose	Sucrose	Maltose	Lactose
Glucose.....	+	-	-	-	-	-
Sucrose.....	+	-	-	+	-	-
Galactose.....	+	+	-	+	-	-
Arabinose.....	+	-	+	+	-	-
Maltose.....	+	-	-	+	+	-
Lactose.....	+	+	-	+	-	+
No sugar added.....	+	-	-	+	+	-

\* Data from thesis by H. Karstrom, *Über die Enzymbildung in Bakterien*, Helsingfors, 1930.

of glucose, fructose, mannose, and sucrose are constitutive enzymes, while those fermenting galactose, lactose, maltose, and arabinose are adaptive enzymes. Note that the galactose enzyme appeared when the cells were cultivated in the presence of lactose, lactose being hydrolyzed with the formation of galactose and glucose molecules, and that maltase is formed in sugar-free broth. Many of the adaptive enzymes appear to be hydrolyases.

This work was soon confirmed and extended to other organisms. In

those instances where enzymes are apparently produced as the result of a specific response to the addition of a particular substrate to the culture medium there are three general explanations. These are (1) adaptation by the selective growth of organisms in the culture potentially capable of forming the particular enzyme, (2) impression of a new but transient character on the cells as an adaptive response to the nature of the environment, and (3) the liberation of a suppressed character. It has now been demonstrated that adaptive enzymes can be formed in the absence of detectable cell multiplication, but not necessarily in the absence of protoplasmic growth or change. Adaptive formation of enzymes might be protoplasmically controlled and therefore not transmitted to offspring produced in the absence of the enzyme-incident substrate. The possibility exists with respect to adaptive enzyme formation that the substrate molecule serves as a pattern or template for the protoplasmic modification of a somewhat similar enzyme or enzyme precursor, or for the production of the enzyme by means of a somewhat labile synthetic system. There are certain analogies in this respect between adaptive enzyme formation and the formation of antibodies in the animal body. It should be borne in mind that adaptive enzyme formation occurs only within certain limits, and these limits may well be genetically controlled.

Adaptive enzymes appear and disappear readily with specific changes in the environment. Other adaptive changes may appear fairly rapidly and readily but frequently tend to remain as a more permanent characteristic of the organism. For example, staphylococci may be quite susceptible to the action of sulfanilamide, but when cultivated in the presence of sublethal amounts of it, they may become highly resistant to this drug. This enhanced resistance has been demonstrated to be due to the production of *p*-aminobenzoic acid by the resistant strain. This apparently enhanced production may simply be an alteration in the metabolic activities of the cell of such a nature as to produce more of this particular substance than is normally involved in the activities of the cell. In many instances this increased productivity is carried through many transfers in sulfa-free nutrient media. In other cases this adaptation to growth in the presence of normally inhibitory or lethal doses of an inhibitory drug is not actually or relatively permanently impressed as a characteristic of the cell. This behavior is of considerable importance in chemotherapy of infectious diseases such as gonorrhea, the gonococcus being susceptible to the action of sulfanilamide. It was believed that the incidence of gonorrhea might be markedly reduced by the therapeutic use of the sulfa drug. A considerable number of cases was treated with success, but in a number of individuals, strains highly resistant to sulfanilamide developed and became disseminated throughout the population, thus reducing the efficacy of this type of medication. This phenomenon of increased resistance or



drug fastness is one of the big stumbling blocks frequently encountered in chemotherapy.

Oddities are at times encountered as the result of the development of enhanced resistance to a chemical agent. Strains of meningococci and other bacteria have developed on exposure to streptomycin which now require small amounts of this drug for growth, the original strains being susceptible to the action of streptomycin. Also, a variant of the mold *Neurospora crassa* has been isolated which requires sulfanilamide for growth and is poisoned by *p*-aminobenzoic acid. Actually the *p*-aminobenzoate is toxic to this variant strain, the sulfanilamide counteracting the toxicity of the former substance.

Many believe that growth in the presence of an inhibitory agent results in the selection of cells which for some reason have an increased resistance to the deleterious agent. Yet increased resistance to one agent does not necessarily imply that the selected cells will have any greater resistance to a second inhibitory agent than that possessed by the original culture. Sometimes increased resistance to related agents is observed, sometimes increased resistance to widely different agents, and at other times specific resistance. It may be that a chance alteration is occurring in the genetic apparatus of a few cells, the resulting resistant forms being derived not from cells originally more resistant than their fellows but from cells which, as a result of a greater degree of plasticity, were able to develop this specific resistance—transient or permanent—by means of some relatively slight change in their genetically controlled metabolism. This may become more evident from a consideration of "biochemical mutants." There are many factors involved, and in time different answers may be found for different observed phenomena of drug fastness. This brief discussion is presented to emphasize that bacteria are susceptible to change and that in our thinking we must consider them as plastic rather than as fixed agents. The power of adaptation is one of the characteristics of life!

**Variation and Natural Selection.** Closely related to but not identical with adaptive enzyme formation is the phenomenon of the natural selection of a strain of a given species best adapted to growth in a particular environment. This type of variation was independently described by Neisser in 1906 and by Massini in 1907 in studies with an organism which in all respects except inability to ferment lactose appeared to be *Escherichia coli*. When this organism was streaked on Endo agar (nutrient agar plus lactose plus fuchsin-sulfite indicator), the colonies which developed in 24 hr. were colorless, indicating that lactose was not fermented. On longer incubation small red knobs (papillae) developed on the white colonies. When subcultures were made from these red secondary colonies to lactose agar, red colonies developed as growth was initiated. If the subcultures to Endo agar were made with cells from the white colonies,

white colonies again developed with subsequent formation of the red secondary colonies on the white ones. Massini postulated that the development of the secondary, lactose-fermenting colonies was due to a mutation of the strain with which he was working, the mutation enabling the organism to take advantage of the lactose in the medium. To this organism he gave the name *Bacterium* (now *Escherichia*) *coli-mutabile*. The lactose-fermenting variant on repeated subculturing in the absence of lactose was still able to ferment lactose, thus indicating that the mutated strain had developed a new transferable characteristic. Reversion to non-lactose-fermenting strains has been observed, but in general the acquired ability to ferment lactose appears to be relatively permanent, just as the ability of the original mutable strain to produce the red variant appears to be inherent in the culture. Numerous examples of variations of this sort have been observed since these early studies of Massini and Neisser.

Lewis, from a detailed analysis of the behavior of *E. coli-mutabile*, concluded in 1935 that the appearance of the lactose-fermenting variant in a lactose-containing medium was due to natural selection working on a spontaneous variation in the culture. When he inoculated decimal dilutions of colonies of his mutable strain from plain agar onto plain agar, he obtained colony counts ranging from 73 to  $354 \times 10^7$  cells per original colony. When these same dilutions were plated out in a lactose-synthetic medium solidified with plain agar (no peptone or beef extract added), the colony counts indicated an initial population of from 2,080 to 6,300 bacteria per original colony. From the results of experiments of this type he concluded that only approximately 1 cell in every 100,000 cells of his strain was a lactose-fermenting variant, actually or potentially. A genetic interpretation of this phenomenon is difficult unless there is some form of fusion in bacteria with segregation of characters from the genetically complex parents. While we do not know the inherent mechanism of the phenomenon, the development of mutable strains is explicable on the basis that the variants are so few in number that they do not appreciably influence the behavior of the culture until the majority of the organisms have ceased growing owing to the depletion of the readily available nutrients. When this occurs, the variants alone are able to continue multiplying, utilizing the sugar or other substance which will support growth of the variant. It could also be possible that all the cells were potentially capable of utilizing lactose if lactose could enter the cells. Hence this phenomenon could be due not to the sudden appearance of a new enzyme but rather to a change in permeability of the cell membrane to lactose. Once lactose enters the cell, it could stimulate the latent ability of the cell to ferment lactose, or to produce the lactose-splitting enzyme. In a medium deficient in a food-stuff utilizable by the majority of

the cells of a species, only those cells would develop which are capable of utilizing the substance with regard to which the organism exhibited variation. Hence the mutant strain would develop and dominate in the environment suitable for its growth alone, a natural selection of the strain best suited for growth in the particular environment.

A similar type of mutation may be observed when a colony well separated from others is allowed to continue growing. Growth is active only at the edge of the colony while the cells in the center of the colony pass into the death phase of growth. Often such spreading colonies will show clearly defined areas or sectors which differ strikingly from the remainder of the colony (see Fig. 14-3). The variant sector differs from the mass of the colony in appearance. This is particularly striking when a colorless mutant develops as a sector in an otherwise pigmented colony. The formation of sectors is explained on the assumption that a cell undergoes a mutation which alters its hereditary characters. This mutant continues to breed true to its new character, and the cells which develop from the original mutant give rise to the sector. A white mutant from an orange micrococcus, for example, gives rise to a white sector in the colony, and cells transferred from the white area to fresh culture media give rise to white colonies. Aging of the colonies appears to be an important factor in the production of mutant strains, since the same variation in pigment formation can also occur in broth cultures. On streak plates prepared from a young broth culture, the colonies appear to be of the same type, while a similar plate prepared from an old culture of the same strain of bacteria may exhibit a variety of colonies. A very odd behavior is noted when well-isolated colonies of *Pseudomonas aeruginosa* are allowed to develop for many days. As the colony ages, the cells appear to form a considerable amount of slimy substance, and when the agar is maintained inverted in a petri dish, the growth tends to slide downward with the production of a stalactite colony.

**Specifically Induced Type Transmutation.** This phenomenon of specifically induced mutation of types is particularly striking and has been studied in considerable detail with certain pneumococci. At the present time, fifty or more types of pneumococci are recognized, the pneumococcus cell apparently being identical in all types with the exception that different types differ in regard to the nature of the capsular substance which each type synthesizes. However, the organism may lose its ability to produce a capsule and reverts to a non-type-specific pneumococcus. Griffith in 1928 observed that a culture derived from a noncapsulated type II pneumococcus was unable to produce an infection in mice, but when this organism and heat-killed type III pneumococci were injected into mice, the mice came down with a pneumococcal infection from which only type III pneumococci could be isolated. The living noncapsulated pneumococcus

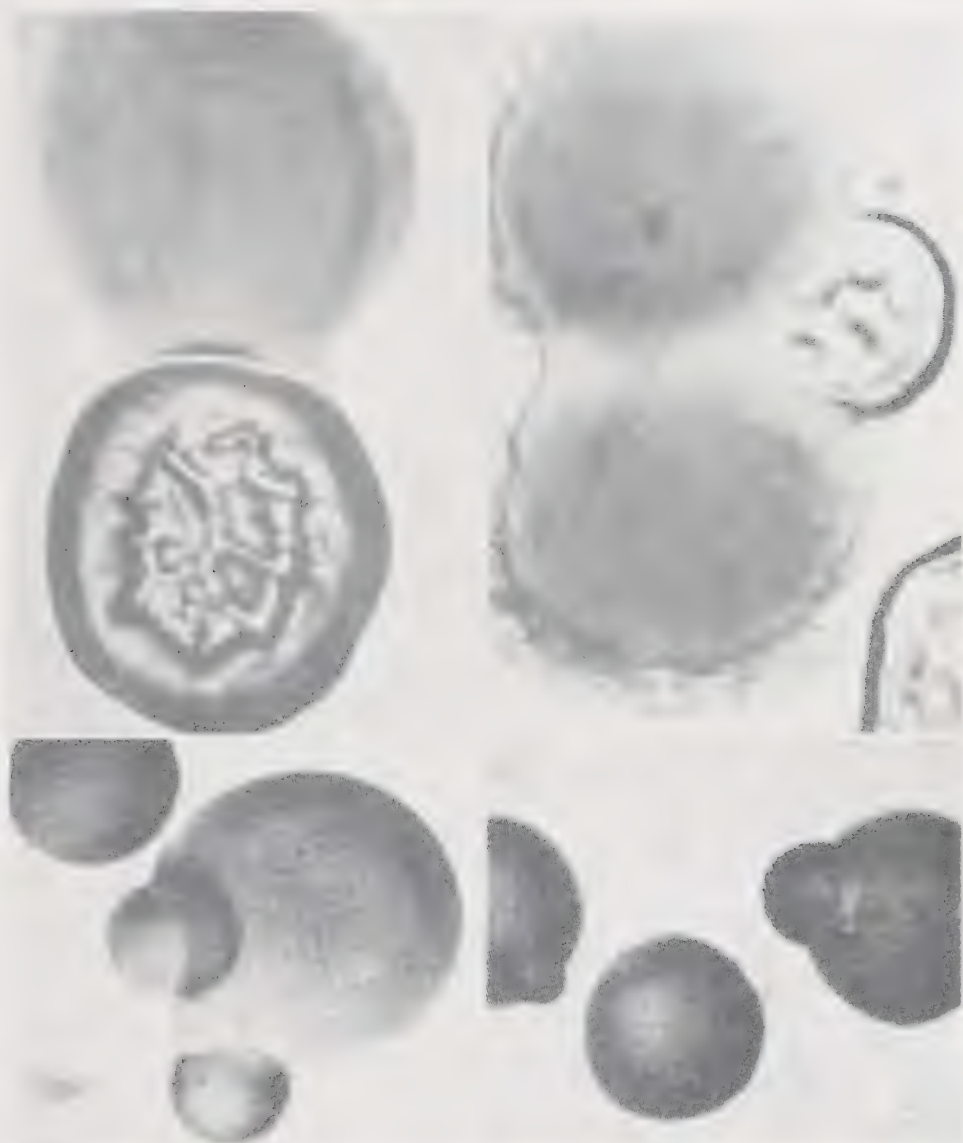


FIG. 14-3 Variation in colony forms of *Pseudomonas aeruginosa*. (Courtesy of E. W. Schultz.)

pneumococci derived from the original type II culture acquired the capsular structure and serological specificity of a type III pneumococcus.

Subsequent observations confirmed this finding and also established that this transformation could be brought about in the test tube by cultivating the nonencapsulated form in a medium containing killed type III cells or extracts of these cells. Later Avery and his coworkers were able to show that this transmutation was induced by a specific chemical agent, a deoxyribonucleic acid produced by the type III pneumococcus. This



specific transmuting agent is active in extremely minute amounts, one part of the agent in over one hundred million parts of broth being able to elicit the specific type transmutation. This same deoxyribonucleic acid can be recovered from cultures of the transmuted type in amounts far greater than were added to the medium and cannot be found in the original nonencapsulated cells derived from the type II pneumococcus. The induced transmutation becomes a permanent characteristic of the induced type III mutant, provided that cultural conditions favorable for capsule formation are maintained. It should be pointed out that the inducing agent, the ribonucleic acid, and the induced type III capsular material, a relatively simple, nitrogen-free polysaccharide, have no chemical structure in common. It is also of theoretical interest that nucleic acids are the chief components of the nuclei of higher forms of life and that bacteria do contain relatively high amounts of nucleic acid. The exact significance of type mutation induced by a specific principle is as yet unknown, but the phenomenon does indicate that a specific chemical agent can elicit a specific mutation with the production of a different type specificity which is spontaneously transmissible in serial cultures.

**Transduction.** This term refers to a phenomenon similar to transformation and implies the transfer of a genetic factor from one cell to another by means of a filtrable agent. The material transferred appears to be composed of deoxyribonucleic acid and the transmitting agent a lysogenic bacteriophage, a phage which is maintained in the host cell but only rarely causes the cell to lyse. Transduction was first observed by Zinder and Lederberg in an experiment in which two species of *Salmonella* were separated from each other by means of a bacteria-retaining filter. It was observed that some of the cells on one side of the filter acquired a property exhibited by the species on the other side. For example, cells of *Salmonella typhosa* acquired the transmissible ability of forming the flagellar antigen of *S. typhimurium*. Transduction, therefore, resulted in the development of a strain having characteristics of both species. Both antigenic and nutritional properties were found to be transducible, apparently at random. Other experiments have shown that only one gene (or equivalent) is ordinarily transmitted to any one receptor cell, that any one gene can be transmitted, and that a gene in the receptor cell is replaced by the transduced one. Transduction has also been demonstrated between strains of *Corynebacterium diphtheriae*, the gene for toxin production being transduced to a nontoxigenic strain. What role the phage particle plays in transduction other than as a carrier for the genetic material has not been determined.

**Bacterial Dissociation.** It has long been observed that a single species of bacteria can give rise to the development of several types of colonies on the same medium (see Figs. 14-3 and 14-4). This variation, commonly

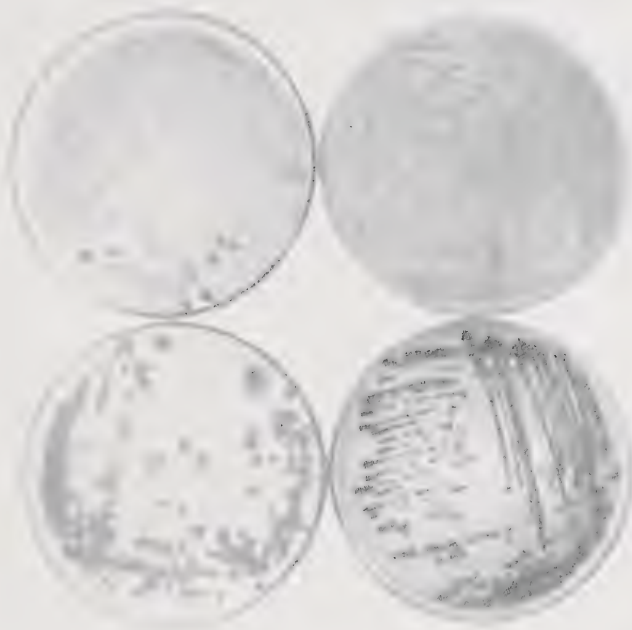


FIG. 14-4 Variation in type of growth observed when transfers are made from different colonies of *Pseudomonas aeruginosa*.

known as *bacterial dissociation*, gives rise to variant strains which exhibit considerable differences not only in colony structure but in cellular morphology and physiology as well, the variant characters tending to be relatively stable. Five general types of variation in colony form, or dissociative phases, have been recognized, together with various degrees of variation between these more distinct phases. The colonies of most bacteria on nutrient agar tend to be round, their edges and surfaces perfectly *smooth* and usually moist and glistening in appearance. This type of colony is generally considered to be the normal form of growth and is known as an *S* (smooth) colony. In those species which exhibit marked capsule formation, the normal type of colony may be rather viscous in consistency, and the colony may appear watery or transparent. This is known as an *M*, or *mucoid*, colony. The cells from *M* colonies are usually very short and show a tendency towards the spherical shape, even in the bacillary species.

When cultures of bacteria which normally produce *S* colonies are cultivated in the presence of specific immune sera or specific bacteriophages, or in the presence of certain salts, particularly lithium chloride, a number of the colonies which develop are frequently irregular in form, their edges are curled or toothed in appearance, their surfaces often dull, dry, and

wrinkled, or *rough*. This type of colony is known as an *R*, or rough, colony. The rough form of colony appears to be the normal form for certain species of bacteria, particularly the sporeformers and the acid-fast bacteria. The cells in *R* colonies appear to be longer and more filamentous than cells from the same species in the *S* phase. The *S*-to-*R* variation can also occur spontaneously in cultures, particularly on aging, while the *R*-to-*S* variation is not observed very frequently except in those species in which the *R* form appears to be the normal form.

Many species may at times produce extremely small colonies which tend to be unstable and which consist of minute cells or structures regarded as *gonidia* by some investigators, hence these colonies are spoken of as *G* colonies. In some species, particularly of streptococci and the tubercle bacillus, *D*, or *dwarf*, colonies are observed which contain diphtheroid cells.

The change in the outward appearance of the colonies is reflected in changes not only in morphology but also in biochemical and other biological properties. When grown in nutrient broths, the *S* forms tend to grow diffusely throughout the medium while the *R* form tends to grow as a film or pellicle on the surface of the broth, or as a sediment at the bottom of the culture vessel. One of the most striking changes is the frequent loss of virulence, i.e., capability of producing infection, when the *S*-*R* dissociation occurs. With organisms such as *Bacillus anthracis* the normal virulent form is the *R* form, the organisms in the *S* phase tending to be avirulent. This behavior, also noted with certain streptococci, is believed to be due to the fact that most normally smooth bacteria produce capsules predominantly polysaccharide in composition, while the normally rough species of bacteria have capsules which appear to be of the nature of a polypeptide, colony appearance being controlled to a considerable extent by the nature of the capsular substance. Although the colony of the normal *R* form may be rough in appearance, other properties of the cells are generally characteristic of organisms normally smooth in appearance. The virulence of a number of pathogenic organisms appears to be associated with their ability to produce a capsule, which in turn may protect the organism to a certain extent against the normal protective forces of the animal host.

Knowledge of these various dissociation phases of the bacteria is far from being complete, and the terminology is somewhat confusing at times. Neither is there any adequate explanation for all the observed phenomena, although the present tendency appears to favor considering the dissociation of bacteria, yeasts, molds, and possibly algae to be caused by accidents to genes. From a normally smooth red bacterium one may observe the formation of rough red, of smooth white, and of rough white dissociates. This suggests that the observed dissociations are due to modifica-



cations of individual genes. Since many of these variations may be reversible, although frequently not readily so, it would suggest that the observed variations are due to a modification or suppression of a gene or genes rather than to a complete loss of a gene or genes. Mutations of many types occur spontaneously at a slow rate, and mutants can be found if we look for them. Their detection is simplified if selective conditions are employed to promote their growth. Much remains to be learned about the genetic structure of bacteria, and a fuller knowledge of hereditary transmission in the bacteria will enrich our understanding of genetics in general.

**Induced Variation in Fungi.** Winge and Lausten demonstrated in 1939 that there is a direct relationship between the possession of particular genes and the ability of the yeast cell to ferment certain disaccharides. A basic implication of this concept is that biochemical defects are not alone in being gene-controlled but that genes are the controlling factors of all the biochemical reactions which the organism is able to carry out. We have considered that with increase in the parasitic nature of a microorganism there is a concomitant loss in synthetic power. What is the mechanism of the losses in synthetic power which have led to the complex nutritive requirements of certain organisms, particularly the pathogens? If the ability to synthesize specific amino acids and vitamins is genetically controlled, it should be possible to take an organism capable of carrying out an essential synthesis and to produce a mutant lacking the ability to carry out the essential synthesis. This has been done particularly well in a series of studies by Beadle and Tatum and their co-workers with the red bread mold *Neurospora crassa* (for life cycle of this organism, see Fig. 14-5).

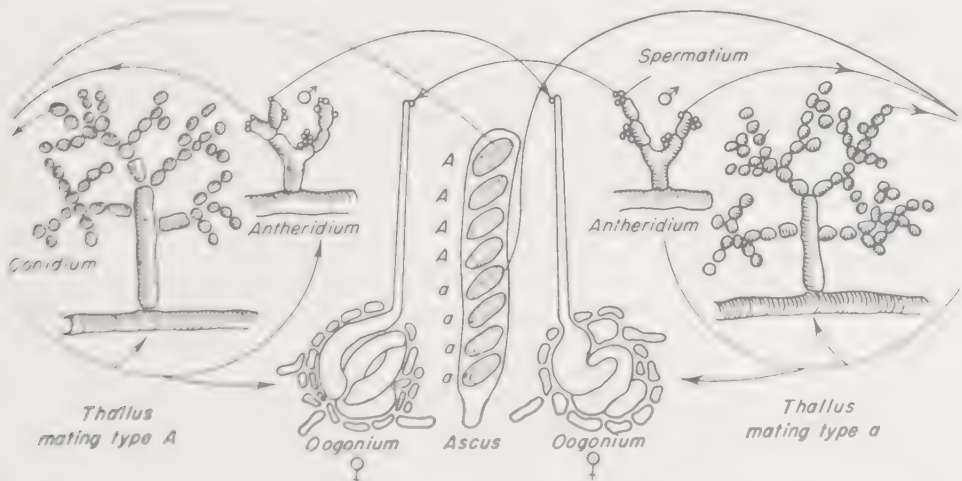


FIG. 14-5. Life cycle of the red bread mold, *Neurospora crassa*. [Courtesy of C. Lindgren, *Genetics of the fungi*, *Annual Review of Microbiology*, 2, 64 (1948).]



*Neurospora crassa* is an ascomycete which grows readily in an inorganic medium containing a suitable carbon and energy source such as glucose and in addition one vitamin, biotin. In this basal medium (minimal medium) *Neurospora* is able to synthesize all of its other organic constituents—proteins containing twenty or more amino acids, at least nine vitamins of the B group, yellow and red pigments, nucleic acid, fats, polysaccharides, and a host of other components. It was known from studies with higher organisms that genes can be inactivated or altered by irradiation with ultraviolet light or X rays, the change produced in the organism when the irradiation was not fatal serving to indicate the function of the gene in the normal organism. Hence, if biochemical reactions are genetically controlled, it then follows that a change induced in a particular gene would result in an alteration in the biochemical properties of *Neurospora*.

Asexual spores of *Neurospora* irradiated with X rays or ultraviolet light were added to a normal culture of the opposite sex or "physiological type." Mycelia of the two types fuse and fruiting bodies form, each ascus containing eight sexual spores. These spores are then transferred separately to a complete medium, i.e., one containing both amino acids and growth factors, and in this manner eight strains derived from single ascospores are established. Asexual spores are then transferred to the minimal medium, and if growth is not observed on the minimal medium, the implication is that a synthetic ability possessed by the original strain has been lost as a result of the irradiation. The nature of the missing synthetic ability must then be determined by a systematic set of tests, since beams of radiation cannot be aimed at a particular gene. Experimentally it has been found that only one or two ascospores per hundred tested give rise to biochemical mutant strains.

Taking a particular modified strain or mutant which cannot grow on the minimal medium, transfers of conidia are made to the minimal medium supplemented with vitamins alone and with amino acids alone, and also to the minimal medium itself and to the complete medium to serve as controls. The experimental procedure to this point is diagrammatically illustrated in Fig. 14-6. In this example, growth in the presence and not in the absence of vitamins implies that the mutant strain has lost the ability to synthesize one or more of the known vitamins. Next, conidia are transferred to a series of tubes of the minimal medium supplemented with individual vitamins. Growth in the minimal medium plus pantothenic acid alone would mean that the mutant had lost the ability to synthesize pantothenic acid. In this manner, forty or more individual mutants were isolated from tens of thousands of ascospores tested, each mutant differing from the others in the loss of some particular synthetic ability.

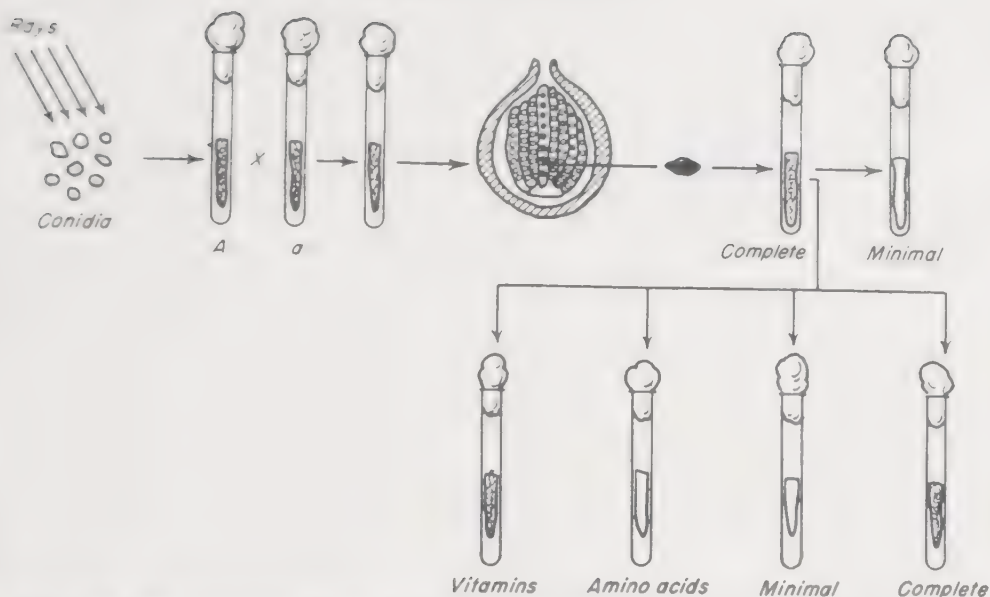


FIG. 14-6. Experimental procedure by which biochemical mutants are produced and detected. [After Beadle, *Genes and chemistry of the organism*, American Scientist, 34, 37 (1946), and by courtesy of Yale University Press, G. A. Batsell (editor), "Science in Progress," 5th Series, 1947.]

Once a mutant is isolated, it must be demonstrated that the loss of synthetic power is associated with genetic structure. This is determined by crossing the mutant strain with the original strain of the opposite sexual type. From the principles of genetics it can be shown that if the two strains differ by one gene, the spore sacs will contain ascospores of two kinds. Four will be like the normal parent (see Fig. 14-7) and not require a particular vitamin (in the above example, pantothenic acid) or amino acid for growth in the minimal medium, while the other four sexual spores from the same ascus will require the particular substance for growth. The two types of spores occur in a regular pattern in the ascus, and from these and other data the geneticist can map the relative positions of the genes on the chromosomes of the organism. Hence, it has been proved that the loss of the ability to synthesize pantothenic acid in the example cited above is due to the loss or modification of a particular gene, and that these syntheses are genetically controlled.

In this manner genes active in the synthesis of vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, niacin, *p*-aminobenzoic acid, inositol, and choline have been demonstrated. Others active in the synthesis of the amino acids arginine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, and valine and in the synthesis of purines and pyrimidines have been similarly demonstrated in mutants lacking specific synthetic powers. Also

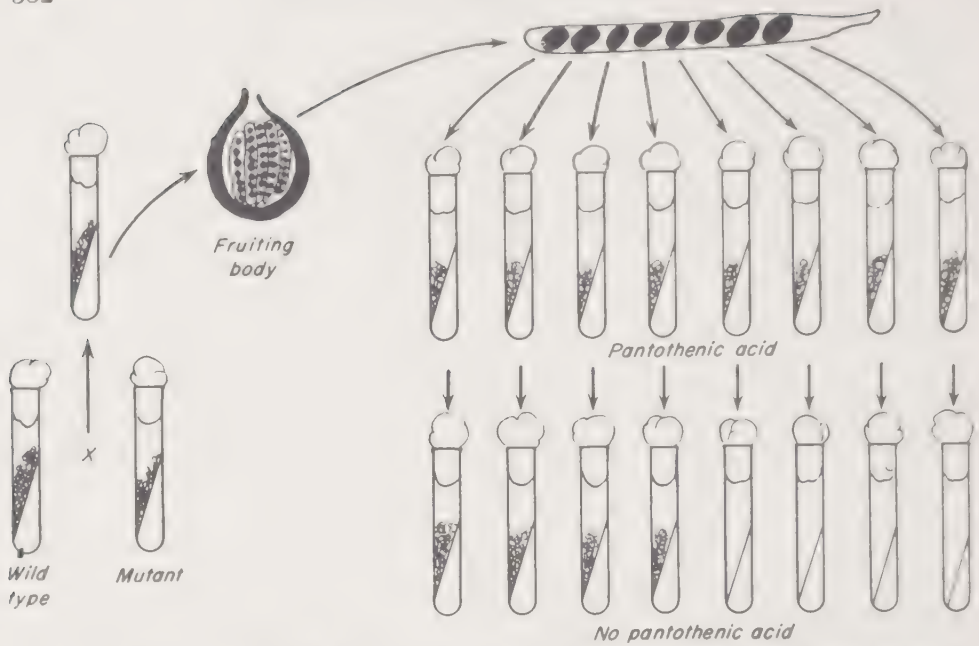


FIG. 14-7. Scheme by which the inheritance of a mutant type is determined. Transfers from the medium supplemented with pantothenic acid to minimal medium are made with conidia. [From Beadle, *Genes and chemistry of the organism*, *American Scientist*, **34**, 40 (1946), and by courtesy of Yale University Press. G. A. Baitsell (editor), "Science in Progress," 5th Series, 1947.]

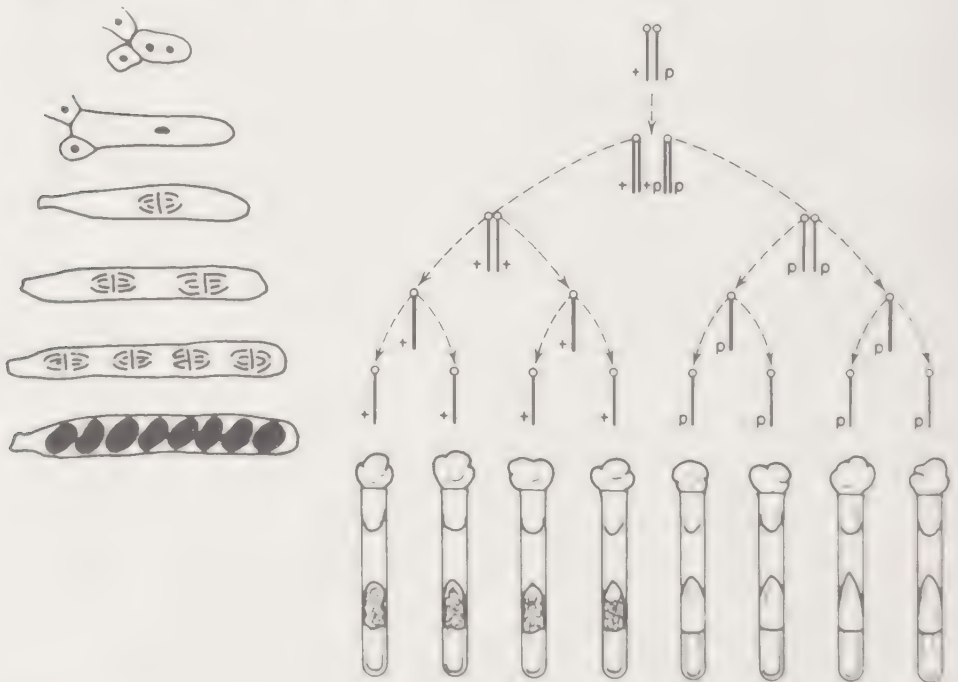


FIG. 14-8. Nuclear and chromosomal basis of genetic segregation in *Neurospora*.



knowledge of the mechanism of certain of these syntheses has been advanced from these studies, and likewise microbiological methods have been developed for the assay of certain of these substances with the aid of specific mutant strains. It should be pointed out that in addition to these biochemical mutants produced by irradiation, other mutations such as variation in the morphology of the fungus, pigmentation, etc., may be produced and observed although these mutations have not been studied to any extent.

Since similar mutants can be isolated following irradiation of bacteria or by treatment with specific chemical agents, it is of interest at least to speculate that a similar genetic mechanism is operative amongst the bacteria. In any event, these studies suggest that the bacterium or higher organism is not a haphazard factory but rather a complex and highly integrated system in which reactions are systematically related in time and space. Alteration or destruction of one gene or controlling factor alters the sequence of events, and the subsequent ramifications may be multitudinous (see Table 14-2). If the single cell, or a higher multicellular form, loses the ability to synthesize a particular substance, it in time ceases to grow as the supply of the substance diminishes. Man, likewise deprived of thiamin, for example, undergoes changes of increasing complexity—inflammation of the nerves, muscular debility, edema, wasting away, and finally death. Again appears evidence of considerable unity in nature!

**Sexual Recombination.** The possibility that sexual reproduction can occur in bacteria has been recognized for a long time, but direct proof, other than pairing of cells under the microscope, was lacking. The development of biochemical markers, mutant strains of *Neurospora* that we have just considered, led to studies of a similar type with bacteria. Tatum and Lederberg obtained mutant strains of *Escherichia coli* which, unlike the original strain, required one or more amino acids or vitamins for growth. When two of these mutant strains were cultivated together in a medium containing none of the required growth factors, about one cell in a million grew and gave rise to a colony, descendants from which, like the parent culture, did not require growth factors. This behavior can be illustrated with a specific example. One strain required biotin (*B*) and methionine (*M*) for growth, the other mutant strain required leucine (*L*) and threonine (*T*). Each could synthesize the growth factors required by the other. Their partial genotypes can be represented as  $B^-M^-L^+T^+$  and  $B^+M^+L^-T^-$ ; the minus sign indicates that the factor is required for growth, the plus sign that it is not. Cells from the colonies that *did* develop were shown to be of the  $B^+M^+L^+T^+$  type, i.e., did not require growth factors. The chance that a cell could mutate with the gain of ability to synthesize both growth factors required by the



TABLE 14-2. TYPES OF BACTERIAL VARIATION \*

<i>Character affected</i>	<i>Variations observed</i>
Size of cells .....	Minute or filtrable (?) forms. Giant cells. Variation in size during period of active growth
Cell morphology .....	Coccoid forms in bacillary species. Bacillary forms in coccoid species. Chains of cells or free cells. Filamentous forms. Involution forms
Staining properties .....	Uniform to irregular. Gram positive to gram negative. Acid fast to non-acid fast
Spore formation .....	Nonsporing variants
Motility .....	Loss of flagella
Capsule .....	Increase or decrease in size. Loss of detectable capsule. Change in type specificity or chemical composition
Type of growth in broth .....	Diffusely turbid to granular sedimenting. Pellicle or no pellicle. Slimy sediment to diffuse clouding
Shape and structure of colonies on solid medium .....	Smooth, rough, mucoid, dwarf, or gonoid. Spreading or raised, discrete. Pigmented or nonpigmented. Secondary papillae on original colony. Nibbled or moth-eaten colonies. Sectors. Variation in optical characteristics
Nutrition .....	Changes in vitamin requirements. Changes in amino acid requirements. Adaption to different energy sources
Fermentation .....	Gain or loss of ability to ferment specific sugars
Miscellaneous .....	Loss of proteolytic power. Loss of ability to produce toxin. Loss of ability to produce hemolysin. Increase or decrease in ability to produce pigments
Virulence .....	Decrease or increase in specific virulence. Shift in virulence for different hosts. Loss of virulence
Antigenic components .....	Presence of flagellar antigen. Group- or species-specific flagellar antigen. Type-specific antigen lost or altered. Shift in somatic antigenic structure
Resistance to harmful agents .....	Variations in resistance with age. Specific increased resistance.

\* Modified from table compiled by Salk in "Fundamental Principles of Bacteriology," 3d ed., McGraw-Hill Book Company, Inc., New York, 1948.

particular mutant is so small that a sexual recombination of genetic material appeared to be the most satisfactory explanation of the observed behavior.

Other workers confirmed the reported behavior and unstable diploid strains were later isolated which threw off haploid segregants exhibiting recombinations of the parental types. These observations, together with the demonstration of linkage groups, constitute good evidence that new genotypes can arise in bacteria as a result of sexual recombination. The phenomenon of mating type has been noted with *E. coli*, types designated as  $F^-$  being able to cross with  $F^-$  types and less readily with  $F^+$  types. The male type transfers only a part of its genetic material to the female, the recombinant inheriting the larger part of its characteristics from the mother cell. Various observations have shown that the two types may lie side by side in conjugation for as long as half an hour, and that during this time only about one-third of the male chromosome enters the cell of the other mating type. Vigorous shaking of the pairs during the course of chromosome transfer will dislodge them, with the result that only a portion of the total genes that can be transferred will enter the female cell. It appears that the chromosome enters the female cell with the same extremity first and with the genes in the same order. The significance in nature of recombination as well as transformation and transduction is not known, but these processes could give rise to new genotypes.

Bacteria as a rule remain relatively unchanged in the laboratory over long periods of time. This results primarily from their being maintained under carefully controlled conditions, and relatively stable strains have developed by selection. Genetic changes do occur in these cultures all the time but only become evident when cultural conditions are changed or when the cultures are exposed to selective agents.

**Population Adaptation.** We have considered that adaptations, either of a temporary or permanent nature, can be observed in pure cultures of bacteria or other microorganisms in the laboratory. Adaptations not only of species but of populations as well are noted under natural conditions and these are of interest and benefit to us. They are generally of a temporary nature, a response to environmental factors, but the organisms that predominate under a given set of conditions have undoubtedly evolved during the past as a result of mutation and selection processes. Population adaptation can be illustrated by observable changes around us.

Leaves, grass clippings, and other organic wastes make up the gardener's compost pile. Sugars and other components leach out and support growth of various microorganisms commonly found under natural conditions. Those that develop most rapidly under these conditions soon outgrow the others, and may be so active that the heat they develop becomes autoinhibitory. Organisms better adapted to growth at an en-

vated temperature then become the predominant population. As the supply of readily utilizable foodstuff diminishes, their growth will be curtailed, the temperature will fall, and other organisms capable of utilizing more complex components of the compost heap will in turn gain the ascendancy. Also the species predominating in any section of the pile will vary from spot to spot depending on the temperature, degree of aeration, concentrations of water and foodstuff, and the nature of the foodstuff available in any one section of this microworld. Some selection of microorganisms would also be brought about by the original nature of the material in the compost heap—different flora developing, for example, in piles of oak leaves, straw, or pine needles. So many varieties of microorganisms are commonly present that it is unlikely that a suitable organism would fail to be present and to develop in a niche best suited for its enzymatic armament. In time much of the material in the compost pile would be solubilized and converted into carbon dioxide and inorganic matter, material suitable for the growth of green plants. The liberation of reduced inorganic matter such as hydrogen sulfide and ammonia would also exert a selective influence by facilitating growth of suitable autotrophic species.

Entirely similar population adaptations occur in the soil, but here algae and protozoa may play a greater role than in the compost heap. A similar situation would prevail in a body of water, but growth of the microbial population would be limited by the lower concentration of available nutrients. Protozoa feed upon bacteria, and the protozoa in turn are consumed by larger organisms in their environment. In the long run the species best suited for a particular environment are selected and maintain themselves in direct competition with other species for the available nutrients, and in competition as well with species predatory upon them in the food chains of nature.

Population adaptations are noted in any mixed population. Algae, bacteria, and protozoa, for example, in sewage oxidation ponds set up a relatively balanced relationship. The algae use carbon dioxide and other waste products of bacterial metabolism for their growth, in turn providing oxygen for continued aerobic respiration of the bacteria. The bacteria may also serve as foodstuff for protozoa, and this aids in keeping the bacterial population below the maximum stationary population level, hence multiplication of the bacteria continues. This picture of events is greatly simplified but is indicative of the over-all influences that are in operation.

Another illustration of population adaptation, possibly limited to bacteria alone, would be that of sludge digestion. Here complex materials are fermented by different species of bacteria with the ultimate production of considerable quantities of carbon dioxide, fatty acids, and alcohols.

The simple acids and alcohols can be utilized as foodstuff by the methane bacteria under anaerobic conditions prevailing during sludge digestion. These substances are oxidized and carbon dioxide is reduced to methane. This utilization of the organic acids and carbon dioxide produced by fermentation prevents the development of acidic conditions, and a population balance is established between the acid-producing and the acid-utilizing species in a continuous-feed digester.

Population balances are also noted if we consider our own microbial population. Certain species are adapted to growth on the skin, on mucous membranes, in the intestinal tract, and so on. Some shift in population balances may be observed if our environment or our food supply is changed. Changes in population are noted following the therapeutic use of antibiotics, in some instances the common inhabitants of the intestinal tract or the ear canal being replaced by pathogenic fungi which may be difficult to replace with the normal nonpathogenic flora. The control of one species often releases that particular environment for occupancy by a different species or group of organisms in either animate or inanimate environments.

During the course of time numerous mutations have occurred in the microbial world, and selection has led to the populations that exist today in the various niches of nature. But a dynamic balance exists, as was briefly considered above, and we can expect that new temporary and permanent population balances will be established in the future as have been done in the past, survival of the fittest being the general rule with microbial populations showing dynamic rather than static behaviors.

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## CHAPTER 15

### BACTERIOLOGY OF SOIL

Soil can be defined as the relatively loose surface material of the earth in which plants grow, in most cases consisting of disintegrated rock with an admixture of organic matter and soluble salts. Originally the earth supposedly consisted mainly of rocks and water, rock being broken down in time primarily by physical and chemical forces, with the formation of smaller particles—thus creating a looser surface layer. Such a milieu would not provide a good medium for the support of most species of microorganisms, but it is known that lichens can grow upon barren rock, slowly converting it into soil. Lichens are symbiotic combinations of a fungus and an alga. The fungus partner makes up the bulk of most lichens, and its hyphae penetrate the substrate upon which the lichen grows. The mold may form acids which slowly decompose the rock, making mineral elements available for its growth. Cells of the algal partner are trapped in the fungal mycelium just below the surface of the lichen, in some instances hyphae of the mold penetrating into cells of the alga. The fungus provides materials for the growth of the alga, the latter in its turn providing organic matter elaborated by its photosynthetic apparatus. Many lichens grow very slowly, but in the course of time they can cause considerable chemical change. As a result of their growth, organic matter is added to the surface layer of the earth, thus providing conditions suitable for the growth of other microorganisms. Many other factors or agents may have been involved in the formation of soil from barren rock, but the activity of the lichens can serve as an example of one biological relationship active in soil formation.

Soils, particularly cultivated and enriched ones, serve as excellent culture media for the growth of many kinds of microorganisms competing with each other for the available nutrient material therein. The microscopic life of the soil is composed of fungi, algae, and protozoa in competition with each other and with larger forms of life also present in the same environment. It has been pointed out (Chap. 12) that soil is composed primarily of inorganic mineral particles which adsorb inorganic salts or ions and organic matter in a surface film of water. This adsorbed film provides an excellent surface for the growth of microorganisms, being

roughly analogous to the surfaces of agar media so commonly employed in the laboratory. It is a medium which is continuously changing in chemical composition, and the organisms growing upon it are subject to marked changes in moisture content of their environment, degree of aeration, and fluctuations in temperature.

A considerable volume of air is present in the irregularities or open spaces between the soil particles. When these pockets are filled with water, the soil tends to pack, dissolved oxygen is rapidly utilized, diffusion of air is retarded, and aerobic conditions give way to anaerobic ones. Normally, aerobic forms predominate in the upper layers of the soil, facultative anaerobes and anaerobes increasing in numbers deeper in the soil. As anaerobic conditions develop in waterlogged soils, a marked shift in the natural balance of population occurs, with fermentative forms increasing in numbers and activity, and the soil becomes "sour," hydrogen ions replacing the metallic ions normally held by the soil particles. Proper drainage, tillage, and the addition of lime are corrective measures for this situation. It is readily apparent that moisture content and degree of aeration are two important factors influencing the ecology of soil. Temperature also plays an important role, microbial activity in the soil increasing as the temperature rises, reaching a maximum dependent on the temperature characteristics of the predominating forms.

Cellulose is the most abundant organic substance continually being added to the soil under natural conditions as the plants upon it die. Other carbohydrates, lignins, proteins, fats, waxes, tannins, pigments, and miscellaneous organic compounds together with their mineral constituents, present in dead plants and to a lesser extent in dead animals and their wastes, also return to the soil from which they were ultimately derived. These materials serve as food for the microbial population, either directly or after serving as food for one organism which is preyed upon by another. Various forms of life in the soil compete with other forms or prey upon them, and the dead bodies of all become food for still other species. Inorganic substances are liberated from organic combination and enter into other phases of the cycles of elements so important in the economy of nature. A continuously shifting balance of population occurs with day-to-day shifts in the nature of the particular environment. This greatly complicates any attempt to develop a complete picture of even the general activities of bacteria in the soil.

The actual numbers of microorganisms in the soil vary markedly with the nature of the soil and the various environmental factors such as moisture, aeration and temperature, as well as with the physical structure of the soil. The numbers of the different groups of the soil's flora and fauna can, therefore, vary from relatively small numbers to staggering totals. A well-cultivated, very rich soil, for example, may contain

as many as 5,000,000,000 bacteria, 20,000,000 actinomycetes, 100,000 mold fragments, 100,000 algae, 1,000,000 protozoa, and small numbers of other microscopic forms per gram of soil, these numbers being determined on the basis of direct microscopic counts. Quastel has estimated that this upper limit of bacterial population is equivalent to over four tons of bacterial substance per acre and that the combined weight of the actinomycetes and higher fungi may approximate this figure. A microbial population of this magnitude, or even a small fraction thereof, would result in an enormous amount of metabolic activity in the soil.

It is difficult to obtain a true estimate even of the numbers of viable bacteria in the soil, any one medium in either a dilution or plate count enhancing the growth of certain forms while inhibiting that of others. Both autotrophic and heterotrophic bacteria are active in the soil, and a medium favorable for the development of one of these nutritional groups is not favorable for the other. It is necessary to use a variety of media in estimating the viable population of the soil, and since many species can grow on a variety of media, the counts overlap each other. Direct microscopic counts are difficult to carry out and do not distinguish between living and dead cells. Counts made in the same area vary from day to day, and vary, when made on the same day, between closely adjacent areas. Daily variations in bacterial counts appear to be related in part at least to fluctuations in the numbers of active protozoa which feed upon the bacteria, the count increasing when any factor diminishes the numbers or activity of the protozoan population.

Soil is ordinarily considered to be primarily a substrate for the growth of plants, and the agriculturist is concerned with improving its value as such a medium. Attempts have been made to study the highly complex interrelationships between the activities of the soil's population and the growth of plants. It is readily apparent that this is a formidable problem, and therefore considerable attention has been directed toward study of the activity of the predominating species in pure cultures, with the hope that these studies can be profitably interpreted to clarify the activities of these bacteria in the mixed population of the soil. This type of study has aided in interpreting over-all changes in the soil, but the struggles for existence between the members of the microbial population can and do influence the behavior of a given species. In addition, higher forms of life, including the plants, during their active life compete with the microorganisms for moisture and essential salts. The root systems of the plants alter the physical character of the soil, thus further complicating this complex biological problem. It is possible to consider here only an over-all picture of the reactions known to play important roles in the maintenance of soil fertility, with emphasis on the activities of the bacteria. Other groups of microorganisms play important roles in

the maintenance of soil fertility, but not necessarily such spectacular roles as do specific groups of bacteria, e.g., nitrogen-fixing species, nitrifying bacteria, and the sulfur bacteria.

### THE CARBON CYCLE

The maintenance of humus and other organic constituents of the soil depends ultimately upon the photosynthetic reactions in the green plant. The carbon cycle was discussed and illustrated in Chap. 8, and a review of this cycle indicates that microorganisms in the soil play an important part in the maintenance of this cycle. They convert organic matter into body substance and at the same time liberate a portion of the carbon as carbon dioxide. If this did not occur, enormous quantities of carbon would be stored in the dead bodies of plants and animals and thus lost to the cycle. Microbic activity is one form of insurance that, in the natural history of events, there will be no major blockage of the carbon cycle as a result of the retention of carbon in organic molecules.

**Humus.** It was mentioned that complex organic matter, derived from dead plants and animals or their wastes, is broken down in the soil by the action of various microorganisms. Cellulose is the main source of carbon and of energy for the microbial population, although actually relatively few species of bacteria can employ it directly. Many species of the higher fungi and certain bacteria, particularly in the genera *Cytophaga* and *Bacterium*, elaborate an extracellular enzyme, cellulase, which together with other hydrolytic enzymes hydrolyzes cellulose to simpler carbohydrates utilizable in the internal metabolism of the cellulose digestors and of other bacteria as well. Other carbohydrates, the proteins, fats, and various constituents of plant protoplasm are also used as sources of building material and energy, while the lignins, pectins, waxes, and related compounds are less rapidly or readily decomposed, and they, or their partial decomposition products, tend to accumulate as a brownish-black complex known as humus.

Humus improves the physical condition of the soil, making it soft and mellow and at the same time increasing the water-holding capacity of soil. It is constantly being formed under normal conditions where vegetation is abundant and is at the same time being subjected to attack by various microorganisms, an equilibrium tending to be established under natural conditions. In cultivated areas, however, this storehouse of food-stuff tends to be depleted with consequent decrease in soil fertility unless at least a portion of some crops is plowed under and manures added to the soil. Soil fertility can in part be restored by the addition of commercial fertilizers, which supply the minerals essential for plant growth but do not maintain or improve the structure of the soil. Restoration of humus



is a slow process but is essential if a proper balance is to be maintained between macroscopic plant life and the microbial activity necessary for the maintenance of a fertile soil. This is another illustration of Le Châtelier's and Fredericq's principle, intensive cropping as frequently practiced altering an essential equilibrium with a resultant decrease in the productivity of the soil as a means of countering the applied force. Humus is composed not of organic matter alone, various salts may be associated with it, in particular iron and some of the elements essential in small amounts for life in general. It is a highly important storehouse and contributes to the maintenance of the carbon and other cycles, serving as a source of materials when other supplies diminish in amount.

### THE NITROGEN CYCLE

In the latter part of the last century, food economists were predicting, on the basis of known supplies of nitrates available for fertilizers, that the world was nearing its capacity for the production of wheat. They predicted that by the 1930's drastic shifts would have to be made in the diet of wheat-eating peoples. It was known that soil fertility could be maintained by means of crop rotation, manuring, and the use of inorganic fertilizers, but practically nothing was known of the enormous activities of the nitrogen-fixing bacteria and of those organisms converting complex nitrogenous matter into ammonia and nitrates assimilable by the plants. Furthermore, chemical methods for the fixation of nitrogen on a commercial scale had not been developed for the production of ammonia- or nitrate-containing fertilizers. Hence the scarcity of known supplies of nitrates and the recognized low concentration of nitrogenous matter in many soils were the criteria employed in the predictions as to future wheat supplies.

The amount of nitrogen assimilated from nitrogen salts in the soil in the United States per year by the green plants is stupendous (probably greater than 5,000,000 tons), and a consideration of the nitrogen cycle well indicates the fundamental role played by the bacteria in making nitrogen available for the green plant, which in turn can serve as a source of nitrogenous matter for the animals.

**Nitrogen Fixation.** This term denotes the conversion of atmospheric nitrogen into compounds in which the nitrogen is combined with other elements. Nitrogen fixation is a part of the nitrogen cycle depicted in Fig. 15-1. Nitrogen fixation can be induced by a limited number of biological agents, or it can be carried out by means of industrial chemical processes. Biological fixation is carried out at low temperatures and pressures, the reverse holding true for the industrial procedures. The actual mechanism of the first stages of biological nitrogen fixation under

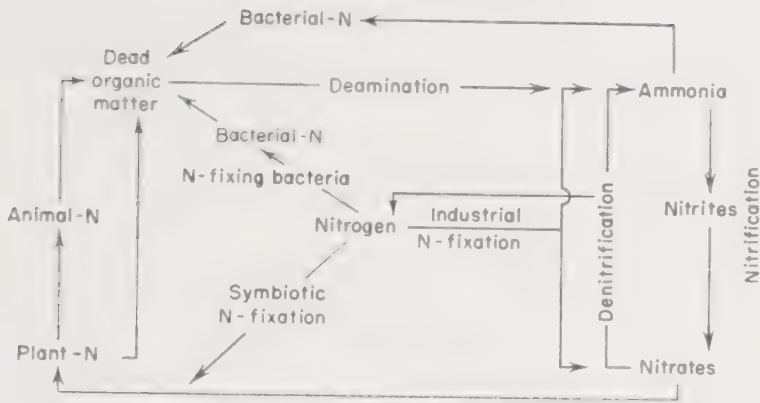


FIG. 15-1. The nitrogen cycle.

the mild conditions existing within the cell is as yet unknown, but progress is being made in the study of the nitrogen-fixation process.

It has been recognized for centuries that the leguminous plants tend to enrich the soil in which they have been grown. Boussingault in 1837 presented evidence which indicated that this was due to an actual fixation of gaseous nitrogen from the atmosphere. His observations showed that there was an increase in the total nitrogen content of soil plus plants above that of the original soil plus seeds when clover was employed as a crop, while the total nitrogen remained constant when wheat was planted. Jodin, in 1862, reported nitrogen absorption when microorganisms from the soil were cultivated in an inorganic medium plus sugar, tartaric acid, or glycerol, and Berthelot in 1885 reported an increase in total nitrogen in potted plants plus soil if the potting soil had not been sterilized. These observations suggested the possibility of the utilization and fixation of atmospheric nitrogen by biological systems, absorption of ammonia from the air being ruled out by control experiments. On the other hand, the measurements were not entirely quantitative in character, and the chemical inertness of nitrogen gas caused many to doubt that nitrogen could be assimilated as such by simple organisms.

In 1888 Hellriegel and Wilfarth demonstrated that plants could develop in sterile sand containing nitrates but that growth was stunted or failed to develop when nitrates were deficient. Addition of soil extracts stimulated growth of legumes (peas) but not that of nonleguminous plants. The increased growth was much greater than could be accounted for on the basis of nitrogen present in the soil extracts, and such growth was always accompanied by the formation of nodules on the roots of the legume. No stimulation of growth, and no increase in nitrogen, was observed when nodule formation did not occur. This led Hellriegel and Wilfarth to conclude that nitrogen fixation is due to the activity of a "soil ferment" present and active in the root nodules. This "ferment,"

*Rhizobium radicola*, was isolated in pure culture by Beijerinck in the same year. Other species of this genus of symbiotic nitrogen-fixing organisms have since been recognized. There is no satisfactory evidence that members of this genus can fix nitrogen by themselves, nitrogen fixation occurring only when the organism is developing in symbiosis with the leguminous plants.

In 1893 Berthelot confirmed and extended Jodin's studies and reported fixation of nitrogen by soil bacteria growing in vitro in media deficient in nitrogenous compounds. He did not isolate and identify the causative bacteria. In the following year Winogradsky isolated an anaerobic bacterium, *Clostridium pasteurianum* (closely related to or identical with *C. butyricum*), which could fix nitrogen by itself in test-tube studies. Beijerinck in 1901 isolated two more free-living nitrogen-fixing bacteria, *Azotobacter chroococcum* (nonmotile) and *A. agilis* (motile). These organisms are aerobes, and the former is of more common occurrence than the latter. *Azotobacter* species and *Clostridium pasteurianum* are widely distributed in soils, are frequently found in water, and are usually associated with other bacteria and with algae (particularly in water). Their activity in nitrogen fixation appears to be enhanced over that observed in pure cultures when they grow in association with other forms. They are most active in neutral or slightly alkaline environments, and their activity appears to be enhanced by humus, probably because of its content of iron, molybdenum, calcium, and other salts.

It has been estimated that under favorable conditions these organisms can by themselves fix from ten to forty pounds of nitrogen per acre per year. The rhizobia are even more active, being capable of fixing, in association with legumes, from fifty to two hundred pounds per acre per year. The total amount of nitrogen fixed per year in the United States by the action of the nitrogen-fixing bacteria, both free-living and symbiotic forms, has been estimated to be as much as one million tons.

Nitrogen fixation is carried out to the greatest extent by the bacteria just considered and to some extent by certain blue-green algae, particularly *Nostoc muscorum*. The algae, due to their particular growth requirements and need for light, are more active in water than in soil. Other organisms may be able to fix nitrogen to a limited extent. It is of interest from the viewpoint of classification that two distinct genera in two families have been established for the nitrogen-fixing aerobes, while the anaerobic species is placed in the genus *Clostridium*, which is composed of a wide variety of physiological types.

The chemistry of nitrogen fixation is obscure, no satisfactory explanation having been advanced as to the actual initial reaction in the fixation process. Vitrano suggests that hydroxylamine ( $\text{NH}_2\text{OH}$ ) is an initial product of fixation by the symbiotic forms, while Wilson and others sup-

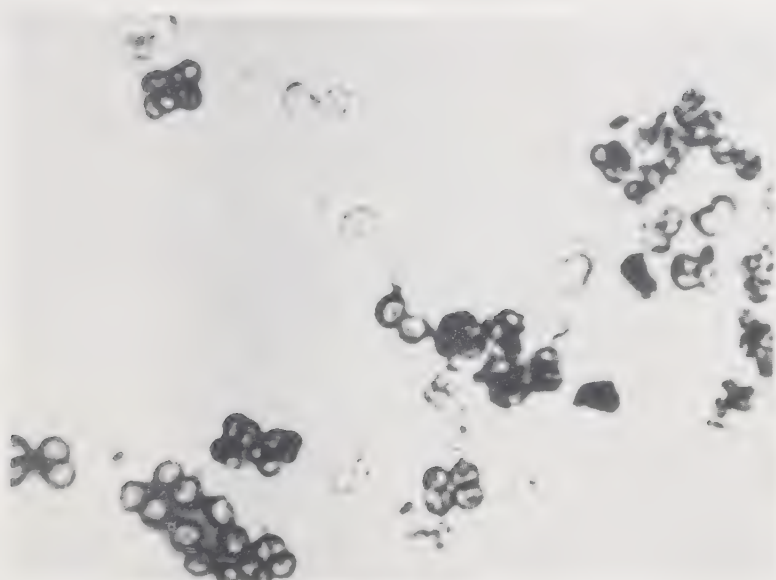


FIG. 15-2. An *Azotobacter* from a colony on a "mud-pie" culture.

post the initial formation of ammonia. Once nitrogen is fixed in combination with other elements, it can then be utilized by pathways common to other organisms. Fixation appears to require considerable expenditure of energy by the cell, being promoted by the presence of utilizable carbohydrates in the test tube or in field experiments. This suggests that carbohydrates could be employed as a fertilizer for nitrogen fixation, the expense probably being a limiting factor in practical application. Nitrogen fixation, on the other hand, is inhibited in soils rich in ammonia or nitrates, the nitrogen-fixing bacteria preferentially employing fixed rather than free nitrogen. The amount of nitrogen fixation, therefore, decreases with increase in nitrogenous content of the soil and is very low in heavily fertilized soils.

**Biological Aspects of Nitrogen Fixation.** The biological aspects of nitrogen fixation, and in particular of the association between the rhizobia and their host plants, are of considerable interest as such while the latter aspect is also of possible significance to medical bacteriology. The general role played by the nitrogen-fixing bacteria has been illustrated in the nitrogen cycle and in the above discussion, and only the symbiotic relationship will be considered here.

The relationship between the legume and the bacterium is analogous to that observed in a localized infectious process, with the exception that both the host and the parasite appear to be benefited as a result of their association. The plant serves as a source of food for the rhizobia and the latter as a source of nitrogenous matter for the plant. In an infec-





FIG. 15-3. Roots of the cow pea: left, uninoculated; right, inoculated with a *Rhizobium*. (From Rahn, "Microbes of Merit," The Ronald Press Company, New York, 1945.)

tious disease the bacteria have to invade the host and establish themselves, in some instances in specific cells or tissues of the host. This is also true of the rhizobia. The rhizobium first of all has to penetrate the exterior coverings of the plant-rootlet cells. There is evidence which suggests that this invasion is aided by a chemical substance produced by the bacterium, apparently the plant hormone indoleacetic acid which causes a deformation of the root hairs when it is present in even relatively minute amounts. The rhizobia penetrate the root hairs at or near the deformations induced by the secreted material, multiply there, and form "infection strands," which penetrate the cells of the cortex. These plant cells are stimulated to abnormal growth and division and form pathological processes or nodules (tubercles) of considerable size (one to several millimeters in diameter). The rhizobia are established in these nodules, and an infection exists, although, as has been pointed out, both the plant and the rhizobia benefit as a result of this association. There is also an analogy between the development of cancers and that of the nodules, a chemical agent initially stimulating abnormal development of the root hairs while other agents may be responsible for the initiation of the abnormal growth characteristic of tumors or cancers. Observations in one field are frequently suggestive for studies in other

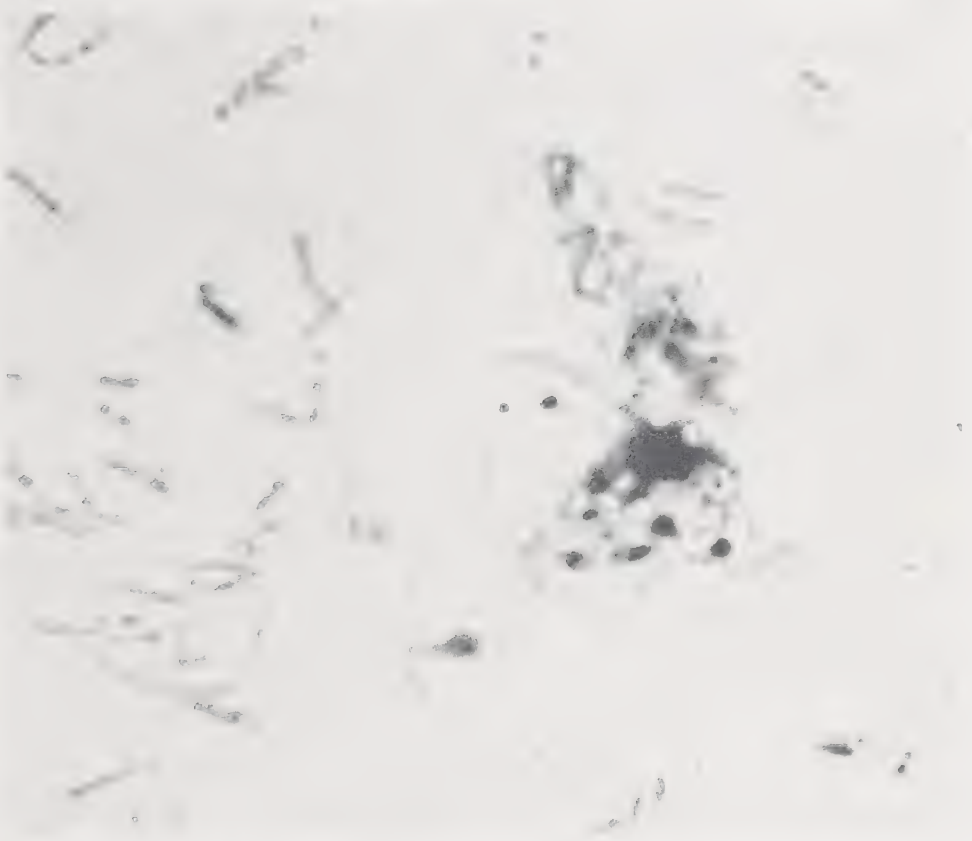


FIG. 15-4. A species of *Rhizobium* from a nodule on the root of a burr clover plant.

fields of research, but analogies must not be taken for facts unless substantiated by concrete evidence.

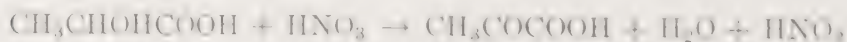
A second resemblance between the legume-bacteria relationship and an infectious process is that a high degree of host specificity exists as far as infectious agents are concerned. This is also true in the legume-rhizobium relationship, strains or species of *Rhizobium* effective for nodule formation and nitrogen fixation in clover frequently being ineffective with other legumes. Other species exhibit host specificity for other legumes, such as alfalfa, peas, or beans. Actually the genus is divided into species primarily on the basis of the most favorable host plants, and the species are named on this basis. It is possible that all the species of *Rhizobium* developed from a common ancestor by adaptation to different host plants. Determination of the factors controlling specificity in one instance might be suggestive in other host-parasite relationships.

Finally, infection of the roots of susceptible plants can spread in a manner analogous to that of an infectious disease of plants or animals.

The rhizobia are frequently spread by means of contaminated seeds acting as mechanical carriers, and in fact this is employed as a method of establishing the rhizobia in a field planted to a legume. The seeds are inoculated with cultures of the specific *Rhizobium*, cultures for this purpose being available from federal, state, and commercial laboratories. Another method of inoculation is to mix the seeds with dirt from a field in which the crop to be planted had previously been cultivated and in which root nodule formation was satisfactory. It should be mentioned that plant pathogens can be disseminated at times by this latter procedure.

**Denitrification.** Denitrification and nitrate reduction in a sense can be considered as the opposite of nitrogen fixation since they tend to reduce the fertility of soil. Nitrate reduction is a reversal of the oxidation of ammonia to nitrates (nitrification) and is accompanied at times by the actual conversion (complete denitrification) of nitrogenous matter to nitrogen gas. Most of the nitrate in natural soils had its origin in the nitrification of ammonia produced from organic nitrogenous matter. Nitrate reduction is an anaerobic process and is of less common occurrence than nitrification, occurring only in the absence of oxygen and in the presence of nitrates and an oxidizable substrate. It occurs to a limited extent in the depths of poorly aerated soils and to a greater extent in sour soils, marshes, and heaps of decomposing organic matter. A considerable number of bacteria, strict anaerobes and facultative ones, are able to reduce nitrates. This reaction is employed in the laboratory as a diagnostic aid for differentiating between various species of bacteria.

The reaction can be illustrated by a consideration of the anaerobic metabolism of an organism such as *Escherichia coli*. In earlier chapters we considered that *E. coli* could develop in an inorganic medium with a single organic compound such as lactic acid serving as a source of carbon and energy, when oxygen was available as a hydrogen acceptor. Under anaerobic conditions, growth does not occur in such a medium unless a substitute for oxygen as a hydrogen acceptor is present. Nitrate will serve this purpose if the enzyme nitratase is produced by the bacterium, this being accomplished by *E. coli* and numerous other bacteria. In the above example lactic acid is oxidized under the influence of lactic acid dehydrogenase to pyruvic acid, the dehydrogenase system transferring two hydrogens to an atom of oxygen made available in nitrate by the action of nitratase, the over-all reaction being



Organisms such as *E. coli* and *Clostridium perfringens*, owing to their possession of a hydrogen-activating enzyme (hydrogenase), can in the



presence of hydrogen gas carry the reaction still further, reducing nitrates to ammonia. Hydrogen in the soil could arise from the fermentation of carbohydrates, and under the acidic conditions prevailing the ammonia would not be lost from the soil to the atmosphere since it could be bound in the form of ammonium salts. Excess ammonia production, however, would result in a loss of ammonia and depletion of the nitrogenous content of the soil. If conditions alter and oxygen becomes available, ammonia and nitrites are oxidized back to nitrates.

A smaller number of species of bacteria, among them being *Pseudomonas aeruginosa*, *Micrococcus denitrificans*, and the autotroph *Thiobacillus denitrificans*, can denitrify nitrates with the production of nitrous oxide or nitrogen, which is lost to the atmosphere. This, therefore, can be regarded as a leak in the nitrogen cycle and can also be considered as a final link in the chain of events from nitrogen fixation to the final liberation of nitrogen.

**Ammonification.** Denitrification (nitrate reduction) serves as one means for the production of ammonia but is of no value to the green plants because the ammonia is produced from nitrates, which are a source of nitrogen for plant life as good as or better than ammonia or its salts. Most of the nitrates in soil are produced from ammonia derived from organic nitrogenous compounds, and the term *ammonification* refers to this liberation of ammonia. The decomposition of proteins via proteoses, peptones, and amino acids has been considered in an earlier chapter, and these reactions take place to a considerable extent in the soil. The amino acids in turn are degraded to simpler units, ammonia generally being formed by deamination under neutral or alkaline conditions and amines under acidic ones. A shift in pH toward the alkaline side frequently results in the oxidation of amines, particularly by *Pseudomonas* species, with the liberation of ammonia. As should be apparent by now, slight shifts in environmental conditions alter the course of metabolic activity in soil or in any medium and unfortunately are too often neglected in our thinking. In soil there are three important variables to consider—the microbe, its associates, and its environment. From the viewpoint of the practical agriculturalist, anaerobiosis leads to decreased soil fertility, aerobiosis to maintenance or improvement of the soil. Any change in the oxygen content results in shifts in the activities and in the numbers of the different species of microbes present in the soil. Once ammonia is formed, it may be directly available to the plant, but generally it is first converted into nitrates by the nitrifying bacteria, aerobiosis being essential for this process of nitrification.

**Nitrification.** Nitrification, as has already been indicated, is the oxidation of ammonia to nitrates by the action of bacteria (see Fig. 10-2). The studies of Schloesing and Muntz and of Winogradsky, reviewed in



Chap. 10, established that nitrification is a biological process and is carried out by a limited number of bacteria, autotrophic and highly specific in character. The reverse process, as has just been considered, is non-specific in character and is carried on by a variety of heterotrophic bacteria.

Nitrification occurs in two distinct steps: (1) the oxidation of ammonia to nitrites, which is elicited for the most part by *Nitrosomonas* (two species) or *Nitrosococcus*, and (2) the oxidation of nitrites to nitrates by the two recognized species of *Nitrobacter*. Other genera—*Nitrospirilla*, *Nitrosocystis*, and *Nitrosogloea*, oxidizing ammonia to nitrite, and *Nitrocystis*, oxidizing nitrite to nitrate—are recognized in the Bergey manual, but less information is available concerning their activities. They are all autotrophs belonging to tribe I, the *Nitrobacteriaceae*, of the family Nitrobacteriaceae (Bergey's Manual, sixth edition).

Nitrification is a process limited to the genera mentioned above and serves as a source of energy for the reduction of carbon dioxide by these bacteria and for their growth. These organisms can be cultivated with difficulty in the laboratory, and their growth is inhibited by the presence of organic matter. They do grow, however, under natural conditions in the presence of considerable quantities of organic matter, and it is possible that the associations encountered under those conditions are in some manner conducive to their growth. They are found widespread in soils and are also active under other conditions, e.g., in sewage-disposal plants. Their activities in nature are of enormous benefit to man, since they aid in keeping nitrogen in circulation in the nitrogen cycle. It is fortunate that the ammonia oxidizers and the nitrite oxidizers are always found in association with each other; otherwise nitrites, which are rather toxic, would accumulate and be harmful to the green plants.

**The Nitrogen Cycle for Bacteria.** We have considered the nitrogen cycle in its broad biological applications. A similar cycle can be developed for bacteria alone, and this is possible with no other group of organisms. The various stages of the cycle may be represented as in Fig. 15-5. Nitrogen is fixed by the nonsymbiotic nitrogen-fixing bac-

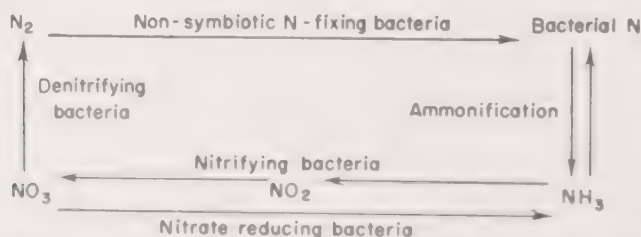


FIG. 15-5. The nitrogen cycle for bacteria. (Modified from Gale, "The Chemical Activities of Bacteria," University Tutorial Press, London, 1947.)

teria and is converted into cellular substance. When these cells die, their protoplasm is available for the growth of species of bacteria capable of utilizing one or more of its nitrogenous constituents, and a mixed population can develop. Ammonia will be one of the end products of the metabolism of these heterotrophic bacteria and can serve as a nitrogen source for still other species of heterotrophic bacteria. This phase of the bacterial nitrogen cycle, therefore, is reversible. With the accumulation of ammonia in the environment, conditions could become suitable for the growth of the nitrifying bacteria, and nitrates would accumulate. Under anaerobic conditions nitrates can act as a hydrogen acceptor and be reduced back to ammonia, a reversible cycle by itself and involving altogether different genera of bacteria, which is not necessary in the ammonia-organic nitrogen cycle. Also under anaerobic conditions, nitrate can be converted into nitrogen in a nonreversible part of the entire cycle by a number of species of bacteria. Only in the Eubacteriales are there organisms capable of carrying out the major steps of the nitrogen cycle. This indicates the widely diverse metabolic types found in this order of living things, and since all of these bacteria can be found in the soil, it also suggests the multiplicity of reactions involved in the maintenance of the dynamic equilibria of microbial populations in the soil.

The carbon and nitrogen cycles are the most important cycles in nature as far as bulk alone is concerned, but the transformations which sulfur, iron, phosphorus, and other elements undergo in nature are equally essential in so far as maintenance of life on the earth is concerned. The principles in all the cycles are the same; the actors and their individual activities differ. Only the two more important cycles, those of phosphorus and of sulfur, will be considered here.

**The Phosphorus Cycle.** Phosphorus is the twelfth most abundant element in the earth, but it is unequally distributed in nature, great deposits occurring in a few localities. Next to nitrogen it is the limiting element of crop production in many soils, and it is also a highly essential element for other forms of life, frequently constituting from 10 to 20 per cent of the inorganic matter of the cell. Within the cell it is found in various combinations ranging from the nucleoproteins to the coenzymes of fermentation and of energy transfer.

Phosphate occurs in the soil in organic combinations and as salts of calcium and other minerals. The mineral phosphates are generally insoluble and are rendered soluble through the activities of bacteria and other microorganisms. For example, the sulfur bacteria oxidize sulfur with the formation of sulfuric acid, which can then react with calcium phosphate, setting soluble phosphate (phosphoric acid) free and at the same time depositing calcium sulfate. Nitric acid (nitrates) or organic acids from fermentation also play a part in the conversion of mineral

phosphates into soluble forms which can be assimilated by the plants. Within the plant, phosphorus enters into various organic combinations in addition to the combinations of phosphates as such. The plant serves as food for animals, and phosphates or organic phosphorus-containing compounds undergo further changes. With death of the plant or animal, or excretion of wastes by the latter, combined phosphorus may once again enter the soil. Phosphates as such are available for microbial or green plant life once again, while the organic phosphorus compounds must be broken down by bacterial action before the phosphorus is again available for plant life. It should be mentioned at this point that the breakdown of nitrogen-, phosphorus-, iron-, sulfur-, or other mineral-containing organic compounds with the liberation of these elements in combinations assimilable by the green plants is spoken of as *mineralization*.

Once phosphates are brought into the soluble form in soil, they can be utilized by the microbial population or by the green plants. A portion of the soluble phosphates, as well as of nitrates, ammonia, and various salts, is lost from the soil by drainage into the streams and larger bodies of water, where these substances become available for aquatic life. Another loss to the soil occurs when plant residues or the wastes of animals and also their dead bodies are not returned to the soil. These substances enter into their respective cycles elsewhere, but their loss from the soil must be made up by the use of appropriate fertilizers.

**The Sulfur Cycle.** No particular genera of bacteria are essential for the phosphorus cycle, and actually it is not as specific as are the nitrogen and sulfur cycles, which have much in common. Nitrogen and sulfur both occur in nature in elementary form, and both enter the atmosphere as a result of combustion or of decay and fermentation. Sulfur compounds, however, are generally present only in traces in the air except around industrial cities or volcanoes. The rains dissolve them and carry them into the soil where they eventually become available for plant life.

Hydrogen sulfide is washed into the soil, and there it is utilized by the autotrophic sulfur bacteria as a hydrogen donor for the reduction of carbon dioxide, sulfides being oxidized to sulfur and eventually to sulfates (sulfuric acid). The sulfur bacteria have been considered under metabolic groups, and therefore the individual species will not be discussed here. Sulfur itself can also serve as an energy source in oxidations carried out by these bacteria and can be employed directly as a fertilizer. These bacteria not only make sulfates available for the green plants, but the sulfuric acid produced as a result of their activities also serves to bring phosphates and other insoluble constituents of the soil into solution and in this manner further increases the fertility of the soil. Sulfur dioxide, as well as hydrogen sulfide, is present in the air, and when it

enters the soil in solution in water, it can be converted into sulfuric acid or sulfates.

Once sulfate is taken up by the green plant, sulfur enters into a variety of combinations, particularly in amino acids and the complexes they form within the cell. Upon death of the plant or of animals feeding upon the plants, much of the sulfur is in organic combination and must be set free from these compounds by bacterial action before it can be assimilated by another plant. This liberation of hydrogen sulfide is frequently employed as a differential test in the laboratory; e.g., *Salmonella typhosa* can be distinguished from *Shigella dysenteriae* by the ability of the former species to form hydrogen sulfide in stab cultures. The sulfur bacteria, both

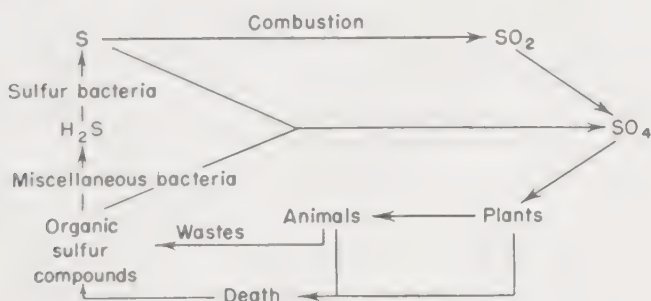


FIG. 15-6. The sulfur cycle.

chemosynthetic and photosynthetic species, are active not only in the soil but also in muds and in sulfide- or sulfur-containing waters, where they also contribute their bit to the maintenance of the sulfur cycle. This cycle is illustrated in Fig. 15-6.

## MICROBES AND AGRICULTURE

We have been considering only one phase of agriculture, the maintenance of soil fertility. The role of bacteria and other microorganisms extends farther than this in modern agriculture, which considers not only the production but also the utilization of the crops. The bacteria in the soil are competing with each other and with other organisms for available nutrients and living room, and while in the over-all scheme of events they do aid in maintaining soil fertility, yet certain of the competitions (e.g., plagues active against rhizobia) may be detrimental to the practice of agriculture. Once more is known of the activities of the different species and genera, it might be possible to control their activities to a greater extent and to direct them along lines most beneficial to man. Certain of the antibiotics produced by the soil forms may in time find as great application in the control of soil bacteria and in particular of bacteria pathogenic for the plants growing thereon as the present appli-



cation of this type of agent to the treatment of human infections. Symbiotic as well as antibiotic effects play important roles in the production and utilization of crops.

Symbiotic relationships are involved in many instances in the development of plants. The combination of a fungus and plant roots to give a structure known as a *mycorrhiza* (fungus root) is essential for the growth of many plants, shrubs, and trees. Many plants do not form root hairs, or form them to a limited extent only, and the root systems are unable by themselves to absorb sufficient water and nutrients from the soil to maintain growth of the plant. Certain fungi grow on the roots, branches of the mycelium often penetrating into the roots, mostly between the cells, forming a network. Other hyphae extend into the soil and garner water and nutrients, some of which are utilized by the plant. The plant in its turn can supply organic matter for growth of the fungus partner. A mycorrhiza is the complex combination of root and fungus which serves as a working unit much like the lichen unit considered earlier in this chapter. Some plants, such as the Indian pipe and certain species of orchids, are entirely dependent upon the mycorrhizal relationship for growth and in fact are devoid of chlorophyll, obtaining organic matter from their fungus partners. In most instances, however, the mycorrhizas are found on the root systems of green plants, truffles being an example of a fungus (highly prized for its flavor) which grows in partnership with certain trees. Most forest trees appear to depend upon fungi on their root systems for a major part of their water and salt supply. This type of relationship is of considerable interest to the biologist and well illustrates a system in which two partners have sacrificed some independence and gained some security in the struggle for existence.

Symbiotic relationships are also noted in the production of meat animals. Cellulose is a major constituent of forage crops, yet cellulose as such is not a good foodstuff, even for the ruminants which consume large quantities of it in their diet. In the cow, for example, there exists in the paunch a mixed microbial flora, bacterial and protozoan, which predigests the cellulose before it enters into the digestive system proper of the cow. Not only do these organisms serve as a source of enzymes for the digestion of cellulose, but they in turn serve as a part of the protein requirements of the animal. Recent studies have indicated that urea can replace much of the protein required by ruminants in their diet, the urea serving as a nitrogen source for the synthesis of the proteins of the microorganisms within the predigestion tract of the ruminants. The microorganisms so produced serve as the protein source for the animal. These studies lend further support to the possibility that in the future microorganisms themselves may to some extent supplant more expensive sources of protein, fats, and carbohydrates in the human diet.

Studies on the relation between virulence of plant pathogens and the genetic composition of susceptible and resistant strains of the plant host may in time lead to marked advances in our knowledge of infection and resistance in man. Agriculturalists have shown that it is possible to develop disease-resistant strains of plants, and this in turn has increased crop yields per acre of tillable ground. Increased yields mean a greater drain of essential elements from the soil and hence that more attention must be paid to the maintenance of soil fertility. There are still enormous losses annually both from plant diseases (see Chap. 24) and as a result of competition between crop plants and weeds, but this is being reduced by the application of methods devised by scientific studies of the various factors involved.

Finally, the residues of many crops are not being returned to the soil and are simply allowed to decompose, either in piles of the material on land or in bodies of water, or the material is dried and burned. In the future these unused end products of agriculture may serve as food for the production of microorganisms which produce materials of more direct benefit to man. Not only is agriculture involved in the maintenance of soil fertility and the production of plant and animal crops, but it is also playing an ever-increasing role in the provision of materials other than foods needed in the daily activities of man. Ultimately the metabolic activities of the bacteria and other microorganisms in the soil control the destinies of man.

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## CHAPTER 16

### BACTERIOLOGY OF WATER

A considerable portion of the earth's surface is covered with water, and while the numbers of bacteria per unit volume are much less than in fertile soils, yet the total volume is so immense that it suffices to support the growth of an enormous number of bacteria, whose activities are of importance in the economy of nature. Various cycles of the elements are operative in aquatic habitats as well as in the soil, and the bacteria play their role in the maintenance of these cycles. Species other than those encountered in the soil are frequently involved; e.g., *Azotobacter agilis* appears to be more important as a nitrogen fixer in water than in soil; but the principles remain the same as those developed in the discussion of soil bacteriology and will not be elaborated further.

Water in general is a relatively poor habitat for the development of most of the saprophytic bacteria since they are adapted for growth in an environment richer in organic matter. The natural flora of a noncontaminated body of water would consist of species capable of growth in dilute solutions of nutrient matter, organic or inorganic in composition. Organic matter would favor the growth of heterotrophic species while a primarily inorganic solution would be most conducive for growth of autotrophic species. An aquatic environment tends to be favorable for the growth of algae, and this growth would pave the way for growth of heterotrophic bacteria in association with the algae or upon their dead bodies. As the algae and other water plants (together with the aquatic animals which feed upon them) die, they settle to the bottom, where they enrich the local supply of nutrient organic matter and provide an environment more conducive to the growth of heterotrophic bacteria. The bottoms of bodies of water rather closely resemble soil in composition, and in this bottom mud anaerobic conditions generally prevail. The flora at the bottom of a body of water, therefore, tends to be quite different from that near the surface. The entire picture can change if considerable quantities of foreign matter enter the water, and it also changes with the changing seasons, bacterial activity being most evident in the summer and early fall months.

The bacterial flora of water tends to be of two general types, the

characteristic aquatic forms (see Fig. 16-1), which have not been studied extensively, and the bacteria which gain entrance to the water from the soil, the air, and in organic wastes. Most studies in water bacteriology have been directed toward the bacteria present in organic wastes, principally sewage, and the role they play in sanitation. The bacterial flora of water is to a considerable extent indicative of the source of the water, and for purposes of discussion natural waters can be divided into four main groups: (1) atmospheric, (2) surface, (3) ground, and (4) stored waters.

**Atmospheric Water.** Rain and snow are the two important atmospheric waters, and they may at times contain considerable numbers of bacteria. These bacteria are primarily those borne by the air which has been "washed" by snow or rain. They are, therefore, most predominant early in the course of the disturbance unless unusual circumstances exist. Leeuwenhoek early observed that rain water tended to be free of microorganisms. It is readily apparent that rain or snow does not provide a suitable pabulum for the growth of bacteria and that the forms present in such waters as they fall through the atmosphere are carried mechanically.

**Surface Waters.** Rain or snow carries air-borne bacteria to the earth, and on the earth's surface the fallen water is immediately contaminated with the soil flora of the area. These organisms are carried with runoff water into the streams which drain the area, or they may be carried with seepage water into the soil to an extent dependent upon the filtration characteristics of the soil. The numbers of bacteria carried in the drainage from the soil tend to decrease with increasing length of the rainfall, and the late runoff tends to dilute the bacterially rich waters previously carried by the streams into their depositories.

Streams originating in high, rocky areas contain relatively little organic matter in solution, and the numbers of bacteria are low. As a

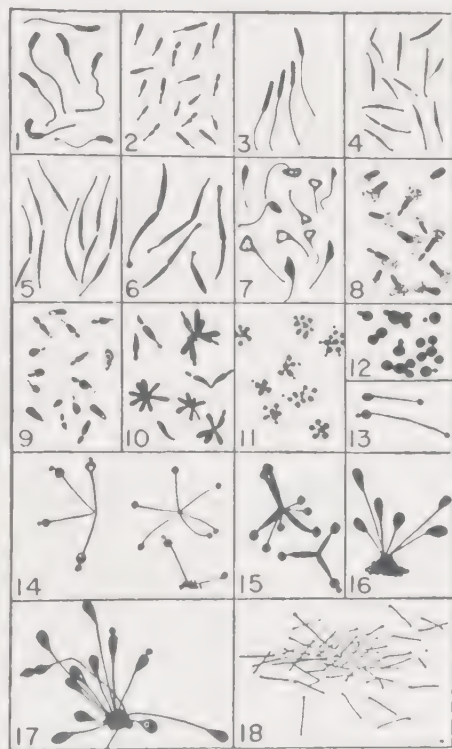


FIG. 16-1. Various species of stalked bacteria from a freshwater lake. [From Henrici and Johnson, *Journal of Bacteriology*, **30**, 61 (1935).]



stream flows through cultivated areas, it receives more and more organic matter and minerals, and the bacterial count increases to a marked extent. Organic and industrial wastes are contributed to streams flowing through heavily populated areas. With the influx of sewage into a stream the bacterial flora shifts from predominantly soil forms to a mixture of soil and fecal bacteria, many of the latter tending to disappear rather rapidly in the struggle for existence in the stream.

The influx of considerable quantities of organic matter into water facilitates growth of the saprophytic species and of protozoa. The latter in turn act as a limiting agent for bacterial growth, as many species feed upon the bacteria. Organic matter and silt in suspension in the water tend to settle out and to carry bacteria and other microorganisms with them. This settling out and partial purification is most pronounced in deep, sluggish streams and enhances growth in the bottom ooze. The presence of organic matter in suspension and in solution in water results in an increased demand for oxygen for respiration of the nonphotosynthetic forms of microbial life and also for the spontaneous oxidation of the organic material. The amount of oxygen required by a sample of water for the oxidation of its organic (and to a limited extent, inorganic) content can be determined by chemical methods and is known as the biochemical oxygen demand (BOD). A water with a high BOD suggests that anaerobic conditions are present, or can develop, and that putrefaction and fermentation will prevail if the water is not continuously aerated. A water low in dissolved oxygen and with a high BOD will be unfavorable for the growth of higher forms of animal life, and in particular this situation is reflected in a marked decrease in the fish population.

Forces other than settling alone and inhibition of growth of aerobic bacteria by decreased oxygen tension are operative in the self-purification of a stream. In turbulent streams there is greater chance for the bacteria to be exposed to the germicidal action of ultraviolet light. Owing to its low penetrating power in water and its relatively low intensity, there is considerable doubt as to the effectiveness of ultraviolet light as a purification mechanism. Another factor to consider is the plant growth, particularly the algae on the banks and, to some extent, the bottom of a stream. This growth tends to absorb bacteria which come in contact with it. However, the bacteria may grow in association with the algae, and when the latter die and break away, the bacterial count of the water is again increased. Competition for survival between the various aquatic forms is of some importance, particularly for the pathogenic forms, most of which do not survive for long periods in ordinary waters. Temperature also plays an important role in determining the numbers and activity of water-borne bacteria. In some streams, particularly heavily polluted ones like the Ganges, the bacteriophage may be active to some extent

and in limiting the numbers of bacteria in the stream. A flowing stream is so subject to change in its composition and the factors involved are so complex that as yet only general statements can be made with safety. Each stream and each change in conditions present separate problems.

**Ground Waters.** Little need be said about ground waters, as they tend to be relatively free of bacteria if they are obtained from some depth. The soil and other layers such as sand and gravel in the earth's crust act as filters, and water flowing through favorable strata in the earth is rapidly purified. This water, however, must be collected in such a manner that no contamination enters it. Dug wells are frequently so shallow that surface water or water from cesspools or other sewage-disposal units is not subjected to sufficient filter action. Intestinal pathogens can gain entrance to such water supplies and the contaminating organisms be transmitted by means of the water, although it does not serve as a suitable pabulum for growth. Water from drilled wells, which extend to a considerable depth into the earth, is generally safe if collected through a tight iron casing. Chemical and bacteriological methods are available for the sanitary analysis of a water supply and should be employed if there is the least doubt as to the safety of a ground water intended for drinking purposes.

Water from springs is an underground water and, if not subjected to pollution, is safe for drinking purposes. Bacteria from the soil do, however, gain entrance to springs, and the bacterial count may be greater than that of water from deep, properly constructed wells. The bacterial flora of unusual spring waters, those high in particular minerals, is of considerable interest to the microbiologist. Sulfur bacteria are frequently found in abundance in sulfur- or sulfide-containing springs, iron bacteria in waters rich in iron, and thermophilic species in hot springs. These organisms for the most part tend to be autotrophic in character since the amount of organic matter in such waters is low.

**Stored Waters.** This term is applied to waters which remain in ponds, reservoirs, lakes, or oceans for some length of time and in which an equilibrium tends to be established between the various organisms constituting the microbial population. Each body of water presents special problems: the nature of the surrounding country and vegetation, the season, individual chemical components, and total inorganic salt content. The general problem of water bacteriology can be well illustrated in a discussion of lake water.

Lakes are generally classified into three main types on the basis of their general suitability for the support of living matter. These are *eutrophic* (well-nourished), *oligotrophic* (poorly nourished), and *dys-trophic* (abnormally nourished) lakes. A eutrophic lake is generally

one which receives water from a stream draining areas rich in vegetation and consequently contains a relatively high concentration of essential minerals and organic matter and is capable of supporting an abundance of aquatic life, both microscopic and macroscopic. Oligotrophic lakes are less productive, usually being spring-fed and therefore of lower nutrient content. Dystrophic lakes drain bogs or swamps and are characterized by a high organic content of peculiar organic matter, generally the remains of partial digestion of matter in the area draining into the lake. These lakes are usually dark in color, the color absorbing sunlight and the water usually being cold a short distance below the surface. The water is usually quite acidic in reaction and is capable of supporting the growth of relatively few species of bacteria or of other forms of life.

Many of the investigations on lake waters have been carried on with the ordinary techniques of soil bacteriology, particularly plate counts in a variety of media and isolation of individual species which will grow on such media. Enrichment media have also been utilized for the study and as an aid in the isolation of individual metabolic groups. It is very difficult to distinguish between soil forms which are usually present in water but are not very active in such a habitat and the species normally active in and characteristic of the water as such. Henrieci and his co-workers have contributed a number of important studies in the field of the bacteriology of lakes, employing a direct microscopic method for studying the bacterial population. They attached glass slides to lines suspended in the water and observed that many unusual species of bacteria (see Fig. 16-1) attached themselves to and grew on the slides. These bacteria, species of *Caulobacteriales* and *Chlamydobacteriales* in particular, could be observed in stained preparations but did not grow readily if at all on ordinary media. They are, therefore, not observed in the usual methods of study. The microscopic method is limited to morphological observations and tells nothing of the physiological activities of the organisms in the water. The increase in number of bacteria per unit area of the slide per day does, however, serve as an indicator of the growth rate of the observed species.

The numbers of bacteria are greatest in the well-nourished lakes, decrease from the shore toward the middle of the lake, and are greatest in shallow areas where plant and animal life is abundant. Henrieci has reported plate counts in a good eutrophic lake ranging from 900 per milliliter in the central part to 30,000 in shallow areas, the microscopic count indicating growth ranging from 500 bacteria per day per square millimeter of immersed slides to 3,500 in the two locations, respectively. The numbers of bacteria developing on a slide per day decrease much more rapidly than the plate count with increasing depth of the lake. Lower temperatures no doubt account for the decrease in bacterial ac-



tivity while the higher plate counts suggest that bacteria developing in the upper part of the water do tend to settle out. Marked changes in counts are observed in the fall and spring months when temperature changes and other factors cause a marked movement (turnover) of water from the deeper portions to the surface. Storms naturally cause a disturbance in lake waters, and violent ones may stir up the more anaerobic species developing in the bottom mud. Seasonal fluctuations in the photosynthetic, microscopic populations and the forms associated with them, in other words, the *plankton*, are one of the most important natural factors influencing bacterial growth in lakes. A high plankton count indicates an actual or potential high organic content of the water and more favorable conditions for the growth of saprophytic species.

The numbers of bacteria in bottom mud approach those in soil and are greatest in the surface layer of the mud. They are probably somewhat less active than in the adjoining soil areas since both the available oxygen and the temperature are generally considerably lower. At the low temperature prevailing in most lake bottoms and under the anaerobic conditions which tend to develop therein, fermentative or incomplete oxidation of foodstuff is commonly prevalent. In shallow areas where sunlight can penetrate readily to the bottom, commensal activity can be observed between aerobic and anaerobic life, and photosynthetic anaerobic species of bacteria, either autotrophic or heterotrophic, can develop. Hydrogen, being an end product of the anaerobic decomposition of carbohydrates and amino acids by a number of bacterial species, is present in appreciable amounts in many muds and can be utilized as an energy source by hydrogen-oxidizing bacteria in the upper layer of mud if oxygen is available. Carbon dioxide is also present in abundance and, as has been shown by the studies of Barker, will act as a hydrogen acceptor for oxidations carried out by the methane bacteria. It was formerly believed that the methane (marsh gas) commonly produced in swamps and in sewage-disposal systems was produced directly from organic matter. Barker, as a result of a variety of studies including the use of radioactive isotopes, demonstrated that organic matter is decomposed by the methane bacteria with formation of carbon dioxide as one end product of decomposition. This carbon dioxide, or that produced by other forms of life, is utilized as a hydrogen acceptor in the metabolism of the methane bacteria and is reduced to methane.

**The Seas.** The general types of bacteria found in salt waters resemble those found in fresh water, many of them possibly having arisen by adaptation of fresh-water or soil species to growth in the presence of considerable quantities of salt. Many of the salt-water bacteria will not grow in media of reduced salt content and are spoken of as *halophilic*, or *salt-loving*, bacteria. Plate counts indicate that bacteria are most



numerous near the shore and particularly near the outlets of streams, although most species of fresh-water-borne bacteria die off rapidly in waters of high salinity. The numbers of bacteria decrease quite rapidly a short distance from the shores of large bodies of salt water, and many of the species in the bulk of the surface water of oceans are associated with the plankton and in particular with the diatoms. Pigmented species constitute about one-fourth of the colonies isolated from fresh water while about three-fourths of salt-water bacteria are chromogenic. The significance of pigmentation is not known. It is also of interest that sea water is the main source of agar-liquefying bacteria and also of the luminescent species.

Studies on the microflora of waters of very high salinity indicate that life can exist under these extreme conditions. Elazari-Volcani has studied the microscopic life in the Dead Sea, a body of water with a salt concentration ranging from about 23 g. per 100 ml. at the surface to a maximum of 33 g. Samples taken from different areas and depths contained a number of species of bacteria and algae. The three predominant morphological types of bacteria required a high salt concentration for growth in various media, i.e., were halo-obligatory species. Two species of red pigmented, facultatively anaerobic, rod-shaped bacteria were frequently observed. Both species reduced nitrates with evolution of gas (denitrification); one was fermentative in character, the other not. Both failed to grow in media of less than 15 per cent salt concentration. A red micrococcus and a rose-colored sarcina were also relatively abundant. The four halo-obligatory species were all gram negative.

Three halotolerant species, organisms capable of growth over a wide range of salt concentration, are common in the Dead Sea. They are all gram-negative rods and are characterized by the formation of blue-brown, grayish-white, and yellowish colonies on peptone agar containing potassium nitrate and different concentrations of salt. Salt-resistant spores were also frequently observed and appeared to be spores of typical soil bacteria.

Physiological types encountered in water of the Dead Sea were denitrifying bacteria, aerobic cellulose fermenters, and fibrinolytic bacteria, while sulfur-oxidizing and glucose-fermenting bacteria were present in the sea-bed mud. A rather rich flora of blue-green, and green algae and diatoms was also observed. The existence and development of bacteria in strong brines is an established fact, but studies on the majority of the species are not complete enough to identify positively all the species isolated. And here again one encounters the problem of what actually constitutes a species of bacteria, for many of the isolated organisms could be well-recognized species which have become adapted to growth in a saline environment.

## BACTERIOLOGY OF WATER SUPPLIES

A number of infectious diseases are caused by agents which enter the body in food or drink and which are eliminated in the feces or urine of the infected person, of convalescents, or of healthy carriers of the infectious organism. The principal water-borne diseases are typhoid fever, caused by *Salmonella typhosa*; the paratyphoid fevers, caused by species of *Salmonella*; Asiatic cholera, caused by *Vibrio comma*; bacillary dysentery, caused by *Shigella dysenteriae*; and amoebic dysentery, caused by a protozoon, *Endamoeba histolytica*. Water containing human or other animal excreta may at times contain one or more of the above pathogenic forms and is said to be *contaminated*. It is generally difficult to demonstrate the pathogens in water, and the sanitarian therefore looks for evidence of fecal pollution, *contamination*, of a water supply. Bacteriological examinations of water supplies deal primarily with determinations of possible contamination of water. Many cities obtain their water supply from lakes or streams, and these waters are usually subject to contamination, to the greatest extent by sewage from other cities farther up the lake or river. It is of considerable importance from the public-health standpoint that drinking waters be made *safe*, i.e., have a good reputation in actual use and yield good results on laboratory examination.

**Sanitary Inspection.** The evidence for possible transmission of a number of infectious agents by water is conclusive. Few would conclude that the water in a stream is safe for drinking purposes if it looks clear but at the same time it is known that sewage empties into the stream at some point above. Likewise the water from a shallow well in a barnyard or close to and below a privy could hardly be regarded as safe for drinking purposes. A sanitary inspection or survey of a water source is simply an attempt to determine whether it is possible for pathogenic organisms to gain entrance to the water supply.

**Chemical Methods.** Chemical methods are of value as an aid in the determination of the past history of the water. They do not differentiate between matter of fecal origin or of origin in the soil or industrial wastes. A high BOD indicates an appreciable organic content and greater chance for survival of pathogenic forms if they are present. An increase in chlorides suggests the possible entrance of sewage, since urine is high in chloride content. The nitrogenous content of the water is most indicative of its past history, as it reflects the nitrogen cycle. Analyses are conducted for combined ammonia, free ammonia, nitrites, and nitrates. Combined ammonia is that capable of being released from organic matter upon proper treatment and generally indicates the amount of nitrogenous matter capable of undergoing decomposition. Free am-

monia is that actually present as such, and a high value is indicative of, but not proof for, recent pollution with sewage. Urea, which is liberated in considerable amounts in sewage, is rapidly broken down into ammonia and accounts for most of the ammonia in polluted waters. The ammonia is oxidized rather rapidly in many waters to nitrites and the nitrites to nitrates. A high nitrite concentration is indicative of fairly recent pollution with organic matter, while a high nitrate concentration is suggestive of more remote contamination. Low values for ammonia and high nitrites or nitrates suggest that many of the pathogenic forms, which would enter if sewage were flowing into the water, have probably been removed or destroyed. Chemical analyses are suggestive of the past history of the water but do not indicate that it is fit or unfit from the viewpoint of the sanitarian. When chemical analyses are carried out at frequent intervals, any abrupt increase in chlorides or in ammonia, free and combined, suggests possible trouble. These analyses are rapid and quite easy to carry out, but bacteriological analyses provide more direct evidence.

**Bacteriological Methods.** Too few pathogenic bacteria are normally present in water contaminated with sewage to enable the bacteriologist to separate and identify them in admixture with other species. *Escherichia coli*, on the other hand, is practically always present in fecal material in large numbers, an average of more than two hundred billion being excreted per individual per day. Besides being present in large numbers in fecal matter, this is the main source of this bacterium in nature. It can be detected fairly readily, even in small numbers in water, and for these reasons *E. coli* is used as an *indicator* of fecal pollution. The presence of this organism in a water supply does not prove that the water is actually unsafe for drinking purposes; it simply means that sewage is entering the water and, therefore, that the possibility exists that intestinal pathogens will at times gain entrance into the water along with *E. coli*. In former practice, water was examined for the presence of *E. coli*, but in more recent years water has been judged on the basis of the presence or absence of "coliform bacteria." The term coliform refers to all species of the genera *Escherichia* and *Acrobacter*. *Acrobacter* species are generally of soil or plant origin, but they are found in approximately 10 per cent of all fecal samples examined. They are somewhat more resistant than *E. coli* to unfavorable conditions in water, and the differentiation between fecal and nonfecal types involves considerable additional time. For these reasons, and for an additional factor of safety, waters are generally examined for the presence of this group rather than for *E. coli* alone. Some workers prefer to examine water for the presence of fecal streptococci or clostridia rather than for coliforms. British pro-

cellures are somewhat different from American ones but serve the same purpose.

As the result of extensive studies, practical experience, and many discussions, standard methods for the examination of water have been recommended and are in common use. These methods are described in detail in "Standard Methods for the Examination of Water and Sewage" published by the American Public Health Association. New editions appear at intervals dependent upon progress in the development of chemical and bacteriological methods for the examination of water. Standards for the bacteriological quality of water have been set up by the U.S. Public Health Service for drinking water provided in common carriers in interstate service. The American Water Works Association has recommended the adoption of these standards<sup>1</sup> by all public-water-supply authorities, but standards in actual use vary in different localities.

In routine bacteriological examinations of water there may be some variation in the actual methods employed, but all operate on the same basic plan. Coliform bacteria ferment lactose with the production of acid and gas, while most other species of bacteria do not utilize lactose or ferment this sugar with the production of acid only. The addition of lactose to nutrient broth tends to promote growth of lactose-utilizing

<sup>1</sup> The minimum number of samples and frequency of testing are dependent on the population served, the number varying from 1 sample per month for a population of 2,500 to 500 samples per 5,000,000 population served. When five 10-ml. portions of a sample are tested, not more than 10 per cent shall show the presence of coliform organisms. When five 100-ml. portions of the test sample are examined, not more than 60 per cent shall show the presence of coliform bacteria. Using a standard technique of water examination, it is possible to obtain an accurate approximation of the most probable number of coliform bacteria per 100 ml. from the following table:

Negative	Positive	Most probable numbers of coliform bacteria per 100 ml.	
		Five 10-ml. portions	Five 100-ml. portions
5	0	Less than 2.2	Less than 0.22
4	1	2.2	.22
3	2	5.1	.51
2	3	9.2	.92
1	4	16.0	1.60
0	5	More than 16.0	More than 1.60

For complete details, see U. S. Public Health Reports, 58, 69-111 (1943).



bacteria in greater amount than that which would take place in plain broth alone; hence the lactose broth serves to some extent as an enrichment medium for the growth of lactose utilizers. Gas production suggests the presence of coliform bacteria. The next steps are the isolation of pure cultures and the identification of the organisms as members of the coliform group.

**The Presumptive Test.** Water, collected in sterile containers and in such a manner that the sample is a truly representative one, is added in measured amounts to lactose-broth (or to other media such as lactose-lauryl tryptose broth) fermentation tubes, aseptic techniques being employed at all times. Production of gas within 24-hr. incubation at 35° C. constitutes a *positive presumptive test*. It is presumptive but not conclusive evidence that coliform bacteria are present, since other bacteria are capable of producing gas. A limited number of species of bacteria by themselves ferment lactose with gas formation. In addition, a number of pairs of bacteria, generally comprised of a gram-positive and a gram-negative species, may elicit the same reaction. One organism produces an acid which is utilized by the second species with the formation of gas. This synergistic action can be prevented in most instances by the addition of an agent such as crystal violet to the broth, the dye inhibiting or preventing growth of gram-positive species.

If no gas is produced in 24 hr., it is necessary to reincubate the lactose-broth tubes for an additional 24 hr. This provides additional time for the development of slow-growing or slow-fermenting strains, or for possible growth of coliform bacteria which had been inhibited by the growth of predominant species in the first incubation period. When no gas is produced during 48-hr. incubation, the test is reported as a *negative presumptive test*, which indicates that the water may be considered bacteriologically safe for drinking purposes. The formation of gas in 24 or 48 hr. constitutes a *positive presumptive test*. Evidence must now be obtained for the actual presence of coliform bacteria.

**The Confirmed Test.** Samples from positive or doubtful presumptive tests are streaked out on lactose-agar plates (Endo or EMB) in order to obtain isolated colonies, or are inoculated into brilliant green lactose bile broth (for details see "Standard Methods"). An indicator of lactose fermentation is present in the agar and serves to differentiate between lactose- and non-lactose-fermenters. Endo's or eosin-methylene blue agar is frequently the medium employed in this step of the examination. If colonies (or cultures) of lactose-fermenting bacteria develop within 24 or 48 hr. and if any are typical of those produced by coliform bacteria, the test is reported as a *positive confirmed test*. Atypical colored colonies are always suspect, and the presence of such colonies would constitute a *doubtful confirmed test*. The lactose differential media actually

differentiate only between colonies of organisms fermenting lactose and those which do not, but experience in judging and differentiating between different types of colonies is of considerable value at this stage of the examination. The confirmed test merely confirms the results of the presumptive test and provides colonies from which organisms can be picked for final identification.

Enrichment and differential media have been employed in the first two steps of the examination and well illustrate the application of physiological principles. In recent years a number of lactose-broth media based on these principles have been proposed for use in place of the differential agar in the confirmed test. The composition of these broths is such that they facilitate the growth of coliform bacteria while inhibiting or preventing the growth of many other species of bacteria. These media are inoculated directly from the original lactose-broth cultures showing either a positive or a doubtful presumptive test. Gas within 48 hr. constitutes a positive confirmed test, and coliform bacteria are assumed to be present. No attempt is made to isolate individual species from the mixture in routine tests since the results of many examinations have indicated that it is not necessary. The use of such media reduces the time and labor required in routine analyses. A medium containing brilliant green and bile in lactose broth appears to be the most satisfactory. Even the most rapid tests require considerable time, and treated waters are generally consumed before the results of a test are known.

**The Completed Test.** The purpose of this test is to prove definitely that the isolated colonies of lactose fermenters do ferment lactose with the production of gas and that this fermentation is induced by typical coliform, gram-negative, nonsporeforming rods. Inoculations are made from at least one typical coliform colony, or two atypical colonies, to plain lactose broth and to agar slants. Gas production in lactose broth and typical gram-negative coliform bacteria on the slants constitute a *positive completed test*, conclusive demonstration of the presence of bacteria of the colon group in the water sample and highly suggestive of the probable contamination of the water with sewage. If necessary, the isolated bacteria can be identified by additional biochemical tests (see Chap. 23).

**Total Count.** Plate counts are frequently employed as an additional aid in the bacteriological examination of water. Such counts are of most value in determining the efficacy of a water-purification measure.

**The Membrane Filter.** Filter sheets (Millipore, molecular, or membrane filter) composed of cellulose esters have been developed that are able to remove bacteria from liquids or from air at a relatively rapid filtration rate. A measured volume of water can be passed through such a filter; the filter sheet can be transferred to the surface of a paper

pad containing a nutrient solution; and on incubation, colonies of bacteria develop on the filter membrane, nutrients diffusing through the filter to the bacteria deposited thereon. The colonies that develop can be counted, a grid imprinted on the filter (see Fig. 16-2) facilitating counting. Total counts can be made in this manner, or differential counts can be made, if a differential medium is employed in the paper base. "Standard Methods," 1955 edition, does not recognize the membrane filter procedure as an acceptable substitute for the lactose-broth dilu-



FIG. 16-2. Mixed culture of coliform and intermediate bacteria from surface runoff water filtered through a Millipore filter. (Courtesy of the Millipore Filter Corporation, Watertown, Massachusetts.)

tion technic described above but does list it as a tentative method for the enumeration of coliform bacteria in water. A preliminary incubation of the membrane filter on an enrichment solution for a period of two hours is recommended before transfer of the membrane to a differential medium. All dark colonies having a yellowish metallic-appearing surface luster on an Endo pad after  $20 \pm 2$  hours at  $35^{\circ}\text{C}$ . are counted as coliform organisms. Transfers can be made to lactose broth if it is necessary to confirm the results. Large or small samples of water can be tested, the volume employed depending upon the number of bacteria expected to be present in the water. The membrane-filter technic is a rapid one and shows considerable promise. Some discrepancies between the most probable number of coliform bacteria as determined by the older method and that determined by the filtration method have been reported, and the latter needs further study.

The membrane-filter technic can also be employed for the evaluation of sanitary conditions of pipelines and other equipment in dairy and

food-processing plants, for the assay of microorganisms in air, for the isolation of specific bacteria from blood and other fluids, and for the removal of the disinfectant in studies on disinfection. Bacteria can be observed directly on these membranes under the microscope.

**Nuisance Bacteria.** Nuisances in water systems and supplies can be created by a number of nonpathogenic bacteria. Nuisances include odor, taste, color production, increased turbidity, slime formation, deposition of materials in the distribution lines thus decreasing their carrying capacity, and corrosion of iron pipes. Slime production is one of the more important problems for the producer and consumer, bad tastes and odors being imparted to the water, making it less desirable for drinking purposes as well as interfering with many industrial processes that require water of good quality. No particular group of bacteria is involved, although the stalked and sheathed bacteria may be of major importance in natural waters. In waters containing wastes such as fibers and other debris, the slime may consist primarily of the debris held together by biological growths. Isolation of bacteria from slime does not prove that the organisms isolated are the cause of the slime since they can be entrapped mechanically. Many of the slime formers are iron-depositing bacteria, either oxidizing ferrous to ferric iron as an energy-providing reaction with precipitation of hydrated ferric hydroxide, or transforming iron indirectly as a result of their metabolic activities. Microscopic examination of slime for the presence of typical iron bacteria is the most reliable method for the detection of true iron bacteria.

Sulfate-oxidizing and sulfate-reducing bacteria also create problems in water systems. The sulfur-oxidizing bacteria contribute to the formation of slime, and those producing a high acidity can accelerate corrosion in pipes. Some of these sulfur bacteria can be recognized by their appearance under the microscope, others by cultural methods. The same is true for the anaerobic, sulfate-reducing organisms. Methods for the detection of nuisance bacteria are described in "Standard Methods." Filtration techniques might prove valuable in the concentration of these organisms, when they are present in small numbers, and for their identification. Methods for the control of the nuisance bacteria are not so satisfactory as those for the control of the spread of the common infectious forms in water.

### PURIFICATION OF WATER

Waters that have been found to contain coliform bacteria to an extent greater than 1 such bacterium per 100 ml. of water are always suspect for drinking purposes. Fewer coliform bacteria than this represent an infinitesimal pollution and an extremely minute possibility of the presence of intestinal pathogens. Experience has shown that in general less



than 1 pathogenic bacterium is present per 100,000 coliforms in contaminated water. Few cities, however, have relatively pure sources of supply of water, and they must resort to purification procedures to render their supplies reasonably free from possible danger of transmission of infectious agents. The ability to dispense a bacteriologically safe water in huge volumes (40 to 200 gals. per capita daily) is a modern miracle of the application of principles of engineering, chemistry, and biology to the needs of everyday life. No other commodity is produced in such volume and in such a high state of purity.

In many municipalities the water supply is sufficiently good that disinfection is all that is required for the provision of a safe, suitable water. In other communities the water source is such that more extensive treatment is necessary to render the water safe and both aesthetically and actually satisfactory for home and industrial use.

**Sedimentation.** River or lake water containing appreciable foreign matter in suspension is allowed to stand in reservoirs to permit the settling out of much of the undesirable material. Many microorganisms are removed during the holding period, and the water undergoes natural self-purification to a limited extent. Sedimentation is frequently aided by the addition of chemical agents (iron or aluminum sulfates, for example) which hydrolyze in water to form flaky, flocculent precipitates which settle out and carry with them much of the suspended matter. By the proper choice of reagents a hard water can be softened at the same time. Sedimentation with coagulation is normally carried out in special coagulation basins where smaller volumes can be handled than in the reservoir proper. Algae, particularly green and blue-green species, frequently give trouble in the reservoirs in summertime. They are not dangerous as such, but their presence leads to the accumulation of organic matter in the water and upon their death bacterial action on their bodies imparts disagreeable odors and taste to the water. Algal growth can be inhibited by the addition of around 5 lb. of copper sulfate per 1,000,000 gal. of water without impairing the quality of the water.

**Filtration.** Since sedimentation and coagulation will not remove all the foreign matter from water, it is generally necessary to pass the water through appropriate filters. As early as 1829 filtration of river water through a sand filter was employed in London in order to remove obnoxious matter. It was observed that this treatment apparently was of value in reducing the incidence of intestinal upsets among consumers of the water, but the germ theory of disease was as yet unknown and the resulting good was ascribed to removal of toxic matter. In the latter part of the last century it was observed that filtration removed 75 to 90 per cent of the bacteria from water. The practical value of filtration became evident in 1892 when a devastating epidemic of cholera broke

out in Hamburg, Germany. Hamburg used raw water from the Elbe River while the city of Altona, adjacent to Hamburg, obtained water from the same source but subjected it to filtration before use. Hamburg had hundreds of cases of cholera per day, Altona few or none. Other examples of a similar nature confirmed the value of filtration of water as a means for the reduction of the incidence of intestinal infections.

There are two common types of sand filters in general use, slow sand or rapid sand filters. The principle is the same in both cases, but in the rapid type, mechanical aids are provided for cleaning the sand *in situ*, and filtration can be maintained at a rapid rate over longer periods of time and with less time out for cleaning the zoogloal debris from the sand. The rapid type of filter is in general use today. Filtration alone greatly reduces the incidence of water-borne diseases, but it is never possible to remove all bacteria in such a rapid and large-scale procedure. It is therefore desirable to disinfect the effluent from the filter beds.

**Disinfection.** Jewell in 1897 added chlorine in the form of bleaching powder as a disinfectant to the filtered water used in Adrian, Mich. Its use as a disinfectant for water supplies spread rapidly. In 1910 apparatus was devised for the mechanical addition of chlorine gas itself to water. Since that time, the use of chlorine or of chlorine plus ammonia for the disinfection of water supplies has been rather universally adopted. Death rates from typhoid fever alone have dropped from 20 or more per 100,000 population to less than 1 in areas such as the Middle Atlantic States where water-purification methods are widely utilized.

It is important to stress again that disinfection is a slow process and that it cannot be speeded up by increasing the concentration of the chlorine in water to much more than 1 part per million, else the chlorine becomes obnoxious to the consumer. Satisfactory results are generally obtained when not more than 0.5 part per million residual chlorine is present after 15 min., and 0.05 to 0.1 part per million at distant points in the water distribution system.

**Purified Water.** Water, after efficient filtration and chlorination, generally has a low bacterial count, and coliform bacteria are no longer demonstrable. If the distribution system is well constructed and maintained, the water on delivery to the consumer should be safe. The use of a common drinking cup or of unsanitary "sanitary" drinking fountains is at times responsible for the spread of infectious agents during the consumption of a safe water. There is no valid reason for the use of a common drinking cup in public places. Drinking fountains constructed with slanting jets so guarded that contamination is almost impossible are available. Faulty plumbing or plumbing fixtures do at times allow water to become contaminated, back siphonage from toilet bowls having been responsible for at least one major outbreak of amoebic dys-

entery. A safe water must be handled at all times in such a manner that contamination is practically impossible.

Outbreaks of gastroenteritis, a mild intestinal disorder, have been traced with reasonable certainty to water that is safe on the basis of bacteriological tests. There is reason to believe that these epidemics may be caused by filtrable viruses. These viruses can pass through filtration plants and are more resistant than bacteria to the action of chlorine. They, or other infectious agents, may also gain entrance to the water in the distribution system. *Endamoeba histolytica* is resistant to chlorination and can be transmitted in sewage-contaminated water which is chlorinated but not filtered. Viruses causing intestinal upsets (and possibly other viruses, e.g., poliomyelitis) and *E. histolytica* must be brought under control in the purification plant before the sanitarian is really pleased.

Not all bacteria are killed by chlorination as commonly employed, and the resistant species, while not pathogenic to man, may be a nuisance in water supplies. In particular, the iron bacteria are frequently objectionable as they can develop in the distribution system and in time deposit such a quantity of iron hydroxide as to block the pipes partially. These and other bacteria following death in the distribution system can undergo decomposition with the development of undesirable odors and tastes in the water. The provision of both a safe water and one of high quality still presents many problems for the bacteriologist, the chemist, and the engineer.

### BACTERIOLOGY OF SEWAGE

Sewage can be defined as *the water supply of a city after use*, or more simply as *dilute water-borne wastes*. It is variable in composition from day to day or even hour to hour and varies considerably between different cities when their industrial wastes are of different characters. Sewage is the water-carried wastes of the laundry, kitchen, and bath plus industrial wastes and also street wastes in cities where there are no separate storm sewers. In a few instances the inorganic wastes create special problems of disposal, but in most instances the main problem consists in the conversion of undesirable organic matter into a state in which it is no longer offensive or dangerous. It is desirable to destroy or remove fecal bacteria at the same time. Safe and efficient disposal of human excreta in sewage is the main goal.

The method best suited for the disposal of excreta is determined largely by density of population and, unfortunately, cost. The outdoor toilet, or privy, is common in rural areas where plumbing is absent or rudimentary, the cesspool or septic tank in more urban areas, and a



Variety of procedures, ranging from dumping into a body of water to almost complete purification, are in use in cities. In any installation two major factors should be (but frequently are not) borne in mind: there should be no opportunity for the sewage to pollute the water supply of the same or of a different home or community, and there should be no opportunity for the transmission of infectious agents from the sewage to man by means of flies or other insects. Biochemical changes occurring in the privy vault, the cesspool, or the septic tank and its larger modifications are similar to those taking place in decaying matter in general. The following descriptions and discussion will be limited to the larger types of installations and to the disposal of human excreta, other materials presenting special problems.

**Purification of Sewage.** Sewage normally is a dilute solution of inorganic and organic matter in water together with an average of approximately 0.26 per cent of matter in suspension (settleable solids). The average adult excretes approximately 68 g. of urea in urine and 20 g. of solid matter in feces per day. The complete oxidation of this material would require about 96 g. of oxygen or 3,200 gal. of water saturated with air, 6,000 to 8,000 gal. giving better results. Complete oxidation is a goal but is never accomplished in even the best sewage-disposal system. Oxidation and other conversions are brought about by the action of a variety of microorganisms, the bacteria playing a very important role. The number and kind of bacteria in sewage vary to some extent, but the chief interest in sewage disposal is in the changes produced by the different bacteria and other microorganisms, rather than in the microorganisms themselves. Saprophytic species predominate, but pathogenic forms are present in sewage and constitute a menace to public health.

Fresh sewage flowing through the mains generally provides aerobic conditions for microbial activity. Urea is rapidly converted into ammonia and carbon dioxide, and if the flow is prolonged, nitrification can occur. The more readily utilized organic compounds may be oxidized to carbon dioxide and water in the mains. When the sewers are carrying capacity loads, anaerobic conditions can develop, and fermentative or putrefactive decompositions become evident.

Most of the organic matter is not dissimilated in the mains and must be disposed of in some manner. When no treatment is employed, the sewage is disposed of by dilution in a body or stream of water, by use for irrigation purposes, or in a few cases by use in fish (carp) ponds. In water the organic matter is oxidized eventually to carbon dioxide and water, but fish and other aquatic life may suffer before this is accomplished since the dissolved oxygen may be depleted. In addition the presence of sewage in a body of water is a definite nuisance. Land irrigated with sewage soon becomes rich in organic matter and unless it



can be kept properly aerated will soon become sour, or "sewage-sick." Sewage farming also can serve as a means for the spread of intestinal pathogens, particularly if leafy or low-growing vegetables are raised on the sewage farm. "Fish farming" in sewage is not a common practice.

The methods commonly employed in sewage-disposal plants involve three main stages: removal of suspended matter, biological digestion, and final treatment. Both physical and biological principles are involved in the disposal of sewage, and the changes accomplished are so complex that only the more general aspects are known with any degree of certainty. A flow sheet for a local installation and one for more complete treatment of sewage are presented in Figs. 16-3 and 16-4.

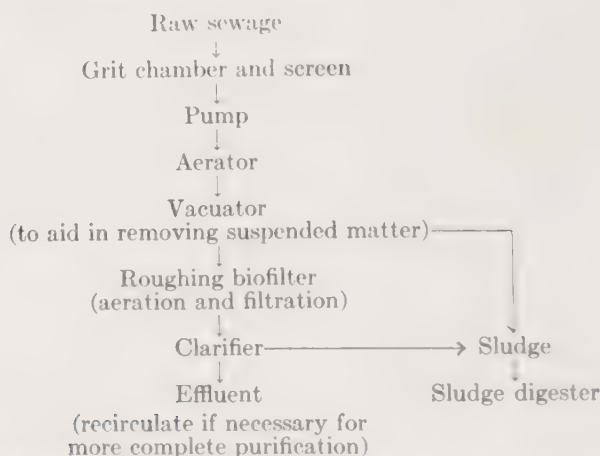


FIG. 16-3. Flow sheet for the sewage disposal plant at Palo Alto, California.

**Removal of Suspended Matter.** Coarse material carried in sewage is frequently removed by screens or strainers in the line at the point of entrance of the sewage into the disposal system. The sewage then passes into sedimentation basins through which it flows very slowly. From 25 to 40 per cent of the organic matter in suspension separates out during the 6- to 24-hr. period in which sewage is normally held in the basins. Many bacteria are associated with the material which settles out of suspension, and this watery complex of inanimate and animate matter is known as *sludge*. Most of the constituents of sludge are not readily decomposed by microorganisms, and the disposal of sludge constitutes one of the major problems in a sewage-disposal plant. Much of the fat and certain other constituents of sewage rise to the top of the sewage in the sedimentation basin, and these substances must eventually be removed and disposed of. In more recent installations filtration through "biofilters" (see Fig. 16-4) is employed after preliminary clarification.

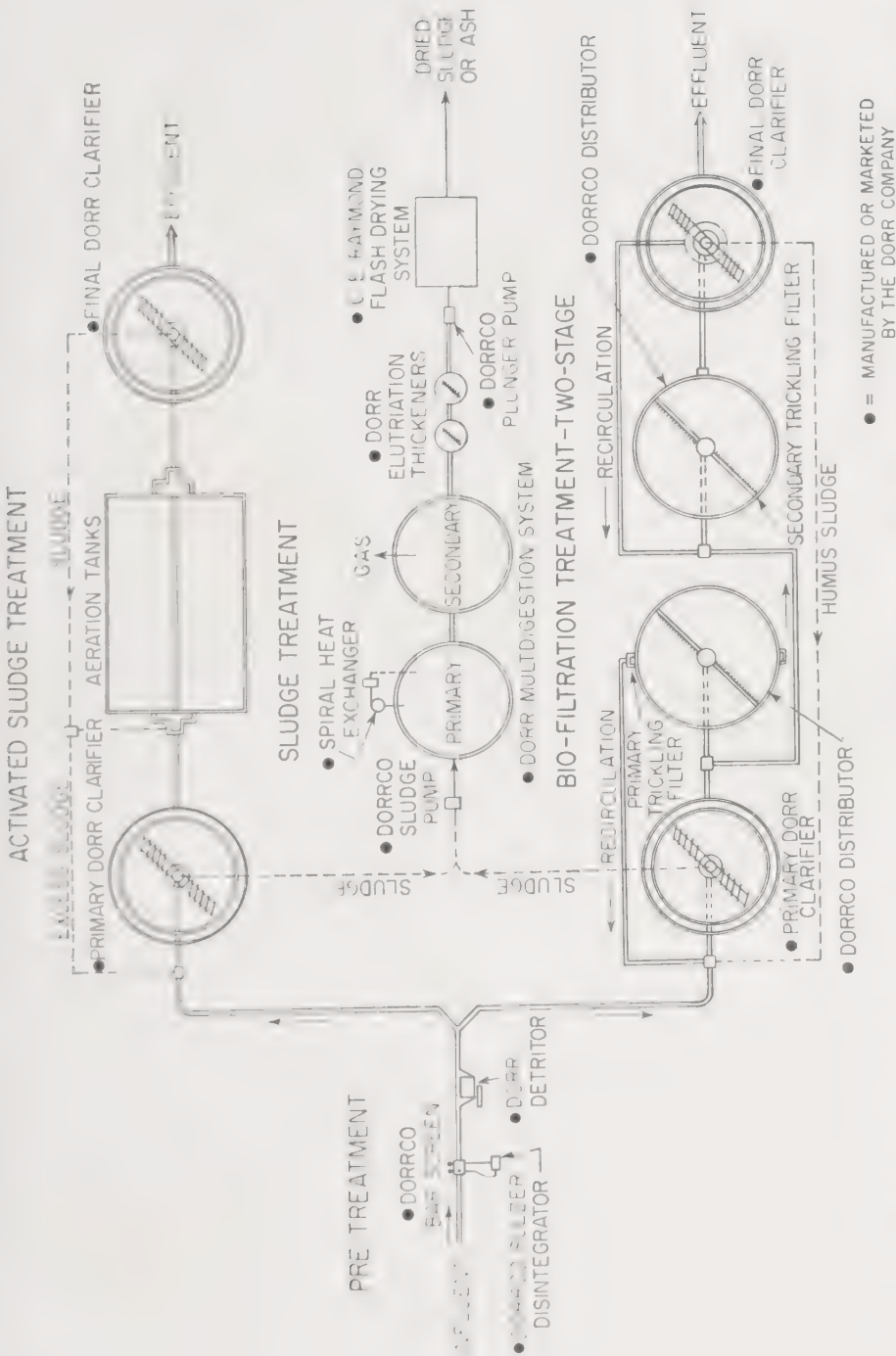


FIG 16-4. Flow sheet illustrating both the activated sludge and the biofiltration treatments for the disposal of sewage. (Courtesy of the Dorr Co.)

**Digestion of Organic Matter in Solution.** In the preceding paragraph the sewage was considered as being held in a sedimentation basin primarily for the removal of suspended matter. Actually much more is accomplished than mere sedimentation since biological forces are extremely active during the holding period and since the sedimentation basin functions as a septic tank. Anaerobic conditions prevail in these tanks, and organic matter in solution is dissimilated with the production

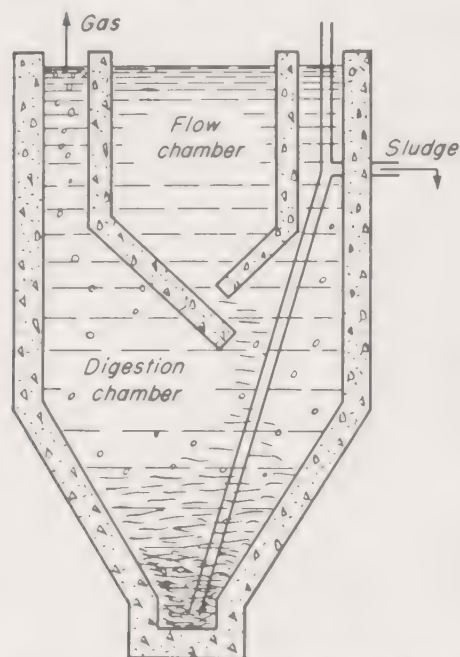


FIG. 16-5. Schematic cross section of an Imhoff tank.

of fatty acids, gases, alcohols, amines, ammonia, sulfides, etc. A portion of the organic matter in suspension is hydrolyzed by extracellular enzymes, and the products of liquefaction then serve as additional food for the microbial population. Nitrates are reduced to ammonia, or denitrification may go as far as the production of nitrogen, reversing any nitrification accomplished in the mains. In this period of intense activity, competition is keen, and many of the pathogenic forms which may be present are destroyed. The less digestible material forms the sludge. Gas formed in these tanks tends to stir up the scum which collects on the surface and also to carry other material to the surface. For these and other reasons, the ordinary

septic tanks have been replaced to a considerable extent by two-story tanks (Imhoff) of special construction.

Sewage slowly flows through the upper story of an Imhoff tank (see Fig. 16-5), and solids settle through slots in the bottom of this chamber into the lower compartment, where further digestion takes place. This type of tank is constructed in such a manner that gas escapes through gas vents without carrying much solid matter upward or disturbing the film which forms on top of the sewage in the upper story. Connections are also provided for the removal of sludge from the bottom of the tank. This type of septic tank is more efficient than the plain type in the removal of suspended matter and in digestion of both liquid and solid material.

In these septic tanks, anaerobic decompositions result in the production of a variety of organic compounds, which must be oxidized further.

before purification is complete. A more recent development in sewage disposal, the activated-sludge process, favors the activity of aerobic bacteria. Sewage flows into large aeration tanks and is inoculated with "ripe" sludge from a previous run. The sewage is continuously and vigorously aerated, and a considerable portion of the readily utilized organic matter is oxidized to carbon dioxide and water. The effluent from an activated-sludge tank is much clearer and contains much less organic matter in solution than that from plain septic or Imhoff tanks. The sludge obtained from the activated process is about four times greater in bulk and much richer in nutrient value than that obtained from septic tanks and has considerable value as a fertilizer when dried.

**Digestion of Sludge.** The resulting sludge from the activated-sludge process of sewage disposal is nearly free from odor, relatively free of pathogenic bacteria, and, as mentioned above, can be dried and used for fertilizer. Drying, particularly in wet climates, may present economic problems. Sludge from septic tanks has much less value as a fertilizer and can be disposed of much more readily by continued digestion. For this purpose it is run into a sludge-digestion chamber, preferably held at an elevated temperature ( $37^{\circ}$  or even  $55^{\circ}$  C.), in which a considerable portion of the material is digested on standing for a period of time. This digestion is an anaerobic process, and considerable quantities of sewer gas (methane) are produced by the action of cellulose-splitting bacteria and similar species. The methane is frequently collected and in some plants is used for heating purposes and even for running the pumping engines. A considerable portion of the sludge undergoes decomposition in the digestion chamber, and the remaining material can be dried and burned or otherwise disposed of. It has little food value for plants but may have some value as a soil conditioner, particularly as an aid in the retention of moisture in sandy soils.

**Filtration.** In many installations the effluent from septic tanks flows directly into a stream or other body of water. When preliminary digestion of the sewage occurs to a sufficient extent, the effluent is generally free of pathogenic bacteria. In many cases, however, it is deemed advisable to chlorinate the treated sewage as a precautionary measure. If the organic content is low, no appreciable difficulty is encountered in the final oxidation of this material in streams, lakes, or oceans if dilution is sufficient to provide a low BOD. This is not always possible, and the effluent can be further purified by filtration before it finally flows from the sewage-disposal plant. Two types of filters are in common use, continuous trickling filters or intermittent ones. In either type the aim is to reduce the organic content of the effluent from the septic tank as rapidly and completely as possible. This is accomplished by aerobic oxidation, organic matter being converted to carbon dioxide and water, am-



monia to nitrates, reduced sulfur compounds to sulfates, etc. Filtration can be employed directly with sewage, or after preliminary clarification, if the organic content is low. These filters frequently are termed "biofilters," since the activities of microorganisms are more important in their operation than is mechanical separation or filtration.

The filter beds are generally composed of coke, stones, or broken bricks in order to present a large surface for the growth of microorganisms. Sand is not used to a great extent as it tends to clog too rapidly. A gelatinous film forms on the filter particles; this film contains enormous numbers of microorganisms, and it adsorbs much of the organic matter in solution in the water being filtered. This material is then oxidized by microbial action. Continuous trickling or sprinkling filters consist of a series of overhead sprinklers through which the preliminary treated sewage is sprayed through the air onto the filter bed. In this step the liquid is saturated with air, and since it trickles through the filter bed, aerobic conditions are maintained. Under ideal conditions the trickling filters could be employed continuously, but in practice it is found that best results are obtained when the filters are periodically rested and cleaned.

The beds of intermittent filters are filled with treated sewage effluent, allowed to stand full for a time, drained, and then allowed to stand empty for another period. Organic matter is adsorbed by the gelatinous film during the holding period, and when the filter is drained, air permeates throughout the bed, and the adsorbed material is oxidized. In the trickling filter, oxidation is a continuous process, while it is a cyclic one in the intermittent filter. Both methods have certain practical advantages and disadvantages. The discharge from properly constructed

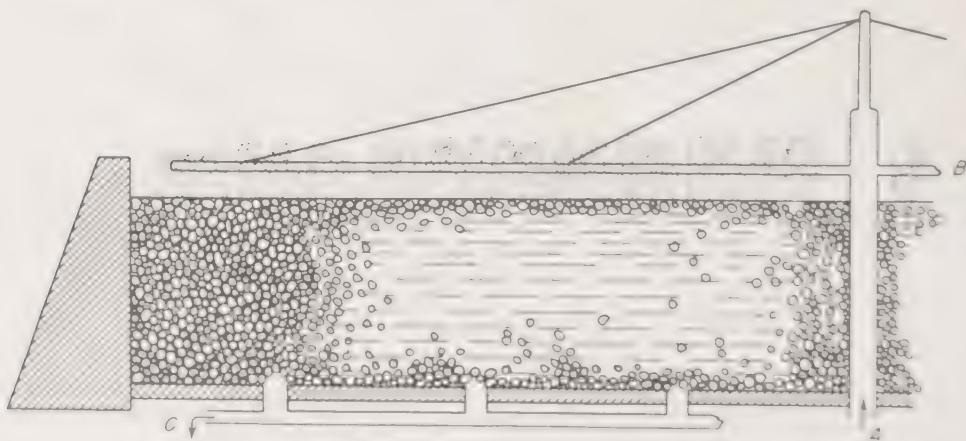


FIG. 16-6. Schematic illustration of a cross section of a circular trickling filter. A is the treated sewage inlet, B the revolving sprinkler arm, and C the drain for the effluent.

filters efficiently operated is of a high quality and with little additional treatment other than chlorination might reenter the water supply of the community from which the sewage was obtained. Such reuse of water may become necessary in large communities in semiarid regions, and the possibility of salvaging water by this means well illustrates what can be done if proper advantage is taken of the metabolic activities of bacteria and allied forms. Actually it is being done all the time in populous areas, except that one city uses the highly diluted sewage of another city rather than its own as a source of water. The cycles of the elements operate to our advantage not only in soil but also in water and water-borne wastes.

**Garbage Disposal.** In some communities garbage is collected and fed to hogs; often it is used to fill in low areas and is covered by a layer of dirt. Methods have been proposed for the disposal of garbage by microbial oxidation of the less stable material in it, the remainder being dried and used as a fertilizer base. Economic factors generally control the choice of methods to be employed in the disposal of such wastes. Microbial disposal of garbage is similar in principle to sewage disposal, with the exception that the material is in concentrated form rather than in dilute solution and suspensions characteristic of sewage.

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## CHAPTER 17

### BACTERIA IN THE AIR

Air, being primarily a mixture of gases, does not provide a suitable medium for the growth of microorganisms. It does, however, serve as a vehicle for the transport of these organisms from one place to another. Almost any sample contains viable microorganisms and inert matter, such as dust particles and pollen grains, in suspension in the air.

Microorganisms gain entrance into the air from a variety of sources, the principal one being dust containing vegetative cells and their spores. The organisms from this source are for the greatest part saprophytes and are therefore of little or no potential danger to man. They can, however, gain entrance to food or drink and cause spoilage. They are also a nuisance in industrial processes such as refining of sugar and can cause serious damage in the course of production of antibiotics and biologicals. Another and more dangerous source of bacteria in the air, particularly under crowded conditions

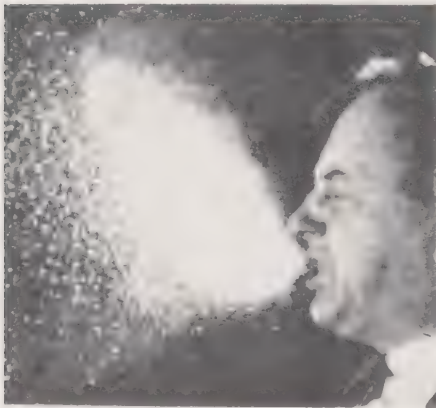


FIG. 17-1. A violent, unstifed sneeze, not quite completed; photographed with the aid of an intense light flash of short duration ( $1/500,000$  sec.) (Courtesy of M. W. Jennison.)

of life, is the upper respiratory tract of man and animals. When an individual coughs or sneezes, an enormous number of tiny droplets are expelled into the air (see Fig. 17-1), and numerous organisms are frequently present in or on these droplets. Normally the bacteria present in the droplets are commensals ordinarily associated with the upper respiratory mucosa and other tissues of the nose and mouth. This flora can be replaced to a considerable extent by pathogenic bacteria or viruses during the course of infections of the respiratory tract, and these infectious agents can be forcibly ex-

polled in the same manner as the more harmless parasites. These pathogenic forms may remain suspended for some time in the air and be

conveyed by means of air currents. Plant pathogens also are frequently transmitted from diseased to healthy plants by means of air currents.

Both the predominant species and the numbers of microorganisms in the air vary somewhat with the locality and with prevalent environmental conditions. Pasteur, in his studies on spontaneous generation, demonstrated the presence of large numbers of bacteria in the air over dusty environments and their relative absence at high altitudes over snow-covered mountains. The numbers present in the air tend to increase during a period of dry weather accompanied by winds, this increase being most pronounced in areas over well-cultivated fields where the soil is finely pulverized and the crops cover only a portion of the ground. Under these conditions the aerobic, sporeforming bacteria tend to predominate, sarcinae and micrococci also being present in relatively high numbers. Molds and yeasts, and particularly their spores, are usually present in considerable numbers, the yeast content of air being greatest around orchard and vineyard country. Rain mechanically removes many organisms and other particles from the air and tends to hold the bacteria in the soil. The population of the air over large bodies of water shifts from characteristic land forms to microorganisms usually associated with an aqueous environment, these latter forms gaining entrance to the air in droplets of spray from the surface of the body of water. The predominant species and the numbers of microorganisms also vary with the altitude above a given area, the actual numbers decreasing with increasing height at which samples are taken. The predominant species at high altitudes tend to be the smaller varieties of bacteria and spores. They have been shown to be present at heights of 20,000 ft., and possibly an occasional one may gain an altitude of many more thousand feet. The germicidal activity of ultraviolet light is high at high altitudes in the clean air prevalent there, and microorganisms have little chance of survival.

**Methods for Enumeration of Bacteria in Air.** When solid nutrient media are exposed to the air, bacteria and other microorganisms will fall on the surface and on incubation will give rise to the development of characteristic colonies. A count of the number of colonies on a plate gives a relative idea of the numbers of viable bacteria and other forms present in the air, but it is of no value for the quantitative determination of the actual numbers present in a given volume. The result obtained when a nutrient agar plate was exposed for 10 min. to the air in a classroom is shown in Fig. 17-2. The use of a variety of media is of value in determining the relative numbers of yeasts, molds, and bacteria.

Three general methods are employed at the present time for quantitative studies on the density of population of air. In one method, a known volume of air is passed through a medium which will retain the bacteria.





FIG. 17-2. Photograph of colonies developing on nutrient agar after exposure for 10 min. to the air in a classroom.

The number of bacteria retained by the medium, generally sand, salt solution, or broth, can then be determined by standard plating and counting procedures. In the second method, the air passes over the surface of nutrient agar, on which the organisms are collected. The colonies which develop on incubation indicate the population density. In one modification of this method the organisms are separated from the air and deposited on the agar by centrifugal force; in another modification the bacteria in a slow stream of air are allowed to settle by gravity onto the surface of agar in a petri dish. Filtration of air through a membrane filter (described in Chap. 16, p. 266) and counts of the colonies which develop constitute a third procedure. All methods and their various modifications have their merits and their disadvantages. The numbers of bacteria per liter of air have been found to range between zero and many thousands. For most purposes the types of bacteria present are of more significance than the total count.

**The State of Suspension.** The majority of microorganisms in the air are seldom present in the free state, generally being associated with suspended particles or droplets. Bacteria-laden dust particles tend to settle out rapidly from quiet air, but they may remain suspended for long periods of time and be transported over wide distances when the air is in motion. Microorganisms suspended as such also tend to settle out of suspension, since they are heavier than air. Since the dust-borne, air-

carried microorganisms are ordinarily saprophytic forms, their presence in air is more of a nuisance than a danger. Forms pathogenic to man are, however, sometimes transported by air-borne dust which has been contaminated with excreta, tuberculous sputum, or other discharges of infected individuals.

Droplet contamination of the air is of much greater sanitary significance than contamination with dust. Pathogenic forms expelled during coughing, sneezing, singing, or even talking can and frequently do remain suspended in the air over long periods of time. Large droplets tend to settle rapidly, but evaporation of water does occur from them, and their size decreases with consequent increased tendency for the residues (droplet nuclei) to remain in suspension in air. These residues are carried in air currents in rooms and other enclosed places and serve as a means of transmittal of agents of respiratory infections.

Instances of the spread of infectious agents in the laboratory have in recent years been traced to the use of pipettes and to other procedures formerly believed to be safe. High-speed photographic methods enabled Johansson and Ferris to demonstrate that minute droplets are formed when the last drop is blown out of the tip of a pipette (Fig. 17-3). Some



FIG. 17-3 The atomization of droplets into the air during bacteriological plating procedures. [From Johansson and Ferris, *Journal of Infectious Diseases*, **78**, 238 (1946).]

of these droplets can gain entrance to the air of the laboratory and from the air to man or other animals. Minute droplets are also produced when dilution blanks are vigorously shaken and can escape from the test tube when the stopper is removed. They may also form in such a simple procedure as pouring from one container to another. These observations indicate that even stricter precautions than were in previous use must be observed when working with pathogenic species.

**Control of Air-borne Microorganisms.** The most important method for the control of air-borne infectious or otherwise undesirable microorganisms is prevention. Any measure which can reduce the possibility for dust to become air-borne or cause it to settle more rapidly will reduce the chances for the dissemination of microbes, spores, or viruses on dust particles. The vacuum cleaner, for example, is of value around the home in so far as it removes contaminated dust from the floor. Its effectiveness is limited by the relatively poor filter action of the collection bag, which may allow some bacteria to pass through with the air expelled from it. The isolation of all individuals having respiratory infections and the use of the handkerchief during coughing or sneezing spells will reduce the incidence of dissemination of the pathogenic forms.

The preventive methods suggested above cannot always be realized, and methods for the destruction of air-borne pathogens are in use, particularly in public places of assemblage and in hospitals or their surgeries. Filtration of air is one important physical method for the removal of matter in suspension in air. Various filter materials, such as wire screens, cheesecloth, and glass wool, are in common use, adhesives at times being added to increase their retentiveness. Actually the cotton plug so widely used in the bacteriological laboratory is a filter. The effectiveness of any air filter depends upon its design, the care which it receives, and the rate of flow of air through the filter. Filters, under normal conditions of use, will not remove all suspended microorganisms, but they do greatly reduce the numbers suspended in circulating air.

It was mentioned in Chap. 13 that ultraviolet light is employed as a physical agent capable of destroying bacteria suspended in the air. The results of ultraviolet-light treatment of air are not very encouraging for common use since many factors influence its action. Some organisms are much more susceptible to its germicidal action than others. The degree of humidity of the air alters the time required for disinfection. A minute film of dust on the light source is sufficient to reduce the output of germicidal rays, and dust and other particles in the air will absorb the light. Theoretically it appears to be a very good agent for the disinfection of air, but the factors influencing its use under ordinary conditions are difficult to control. Opinion concerning its efficacy as a practical control measure is still in a state of flux.



Chemical agents have long been employed for the disinfection of air. Fumigation with agents such as formaldehyde has long been practiced for the disinfection of the air and objects in a closed room. It is effective if properly controlled, but formaldehyde and similar agents, because of their irritating or toxic properties, cannot be employed directly in the air which we breathe. In air of relatively high humidity, hypochlorites or hypochlorous acid in concentrations of 1 part in from 1 to 10 million parts of air have been found to be rather effective bactericidal and viricidal agents. Their activity rapidly decreases when the relative humidity falls below 50 per cent. These agents must be employed in the form of mists (aerosols) or vapors to be effective over a long period of time. Propylene glycol and related glycols have also been found to be active against bacteria and viruses when the chemicals are sprayed as aerosols into the air. One gram of the vaporized material in 2,000 to 4,000 liters of air of 45 to 70 per cent relative humidity and at temperatures below 30°C. is apparently effective and at the same time has little or no toxic activity against man. The action of the glycols has been explained on the basis of their water-absorbing properties. Glycol molecules are deposited or condense on bacteria suspended in the air, and as their concentration increases, they exert marked dehydrating action on the bacteria, eventually destroying them. It should be noted that these agents are not particularly bactericidal or viricidal in aqueous solutions, their activity being dependent to a great extent upon a combination of physical factors.

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## CHAPTER 18

### THE PREPARATION AND PRESERVATION OF FOOD

Man and microbes can use the same foodstuffs in their diets, and a continuous competition for this food exists between man or other animals and the various microorganisms, in particular the yeasts, molds, and bacteria. All foods, except those recently cooked or sterilized, generally contain or are externally contaminated with microorganisms. Most of these are saprophytic forms which so profoundly alter the food as to make it undesirable for consumption, either aesthetically or nutritionally. A limited number of species when properly controlled can bring about desirable changes, while an even smaller number engender end products of metabolism which are toxic to man and other animals. In order to maintain food supplies sufficient to feed the enormous human population, man has been forced to devise methods for the prevention of food spoilage and to improve to some extent upon methods employed since early days for the production of desirable microbe-induced changes in certain foods. It may well prove true that in the not too distant future, microorganisms themselves will become part of the diet of man.

Whether or not spoilage occurs, and the type involved, depends upon the moisture content and the nature of the food, the temperature, and the nature of the organisms present. Yeasts and molds will grow in or on foods having a moisture content as low as 10 to 15 per cent, while bacteria generally require at least 20 to 25 per cent water and generally more than this amount. There are exceptions to such general statements, but it is common experience that relatively dry materials such as hay, grain, thick-rinded fruit, and relatively inert matter such as paper or leather are subject to attack by the higher fungi, particularly in a humid environment. The higher fungi may also break down cellulose walls and pave the way for invasion by bacteria into the interior of the material, where the water content is higher.

The nature of the food is of considerable importance as a determining factor for the microbial flora which will develop in a foodstuff, assuming that yeasts, molds, and bacteria are all present. Yeasts and molds grow most readily in acidic foods of relatively high carbohydrate content, sugar in particular being conducive to the growth of yeasts. The molds are

acidic organisms and tend to grow on the surface, while the facultatively anaerobic yeasts can grow throughout the material. An acidic environment is usually inimical to the growth of bacteria, and acidic foods, e.g., most fruits, which also are rich in carbohydrates, are most subject to spoilage by yeasts and molds. The proteolytic bacteria predominate in the spoilage of foods, such as meat, which are richer in protein content and more neutral in reaction. The predominating types of organisms and the particular species involved may vary during the course of the decomposition (see Influence of Medium on Bacterial Flora, Chap. 11). The temperature at which the material is held also exerts a profound influence (see Chap. 12).

### MILK

Milk may be regarded as a "Dr. Jekyll-Mr. Hyde" type of food. Milk is the most nearly complete food for the majority of animals, particularly during their infancy, but it is also an excellent pabulum for many species of bacteria, including a number of species pathogenic for man. It has been estimated that before the advent of pasteurization and sanitary methods for handling milk, approximately 50 per cent of infants raised on cow's milk died before the age of one from infectious diseases, the causative agents of which were transmitted by polluted milk. Milk is also an exceedingly complex mixture with a rather unstable, sensitive physicochemical structure. Careful handling is necessary to preserve that structure and the nutritive values dependent upon it. Even bringing milk to a boil may diminish its food value, and milk is therefore pasteurized at a time and temperature which will destroy all pathogenic bacteria apt to be present in milk with the least possible change in its characteristics. This must be done on a large scale in creameries, and efficient methods have had to be developed for handling the annual production of more than 15 billion gal. in this country alone.

Milk contains proteins, casein, globulin, and lactalbumin; carbohydrates, principally lactose; fat; different vitamins; salts; and traces of other materials dissolved or in colloidal suspension in water. Milk is nearly neutral in reaction and is a highly suitable medium for the growth of bacteria but is less favorable for yeasts and molds, the latter organisms frequently developing following the growth of acid-producing bacteria.

#### Metabolic Changes in Milk

Five main types of metabolic activities of bacteria in milk can be readily recognized with the aid of an indicator system such as litmus or methylene blue plus chlorophenol red. These indicators indicate

changes in both pH and oxidation-reduction potential. The five types of reaction observed arise from the following groups of bacteria.

1. Bacteria which ferment lactose and form acids to such an extent (pH 4.6) that the casein is coagulated (curd formation). The liquid (whey) is generally expressed during curdling and is watery in appearance.
2. Bacteria which ferment lactose but with less acid production than in 1. No curd or a very soft curd is formed.
3. Bacteria which produce little or no visible change in the milk.
4. Bacteria which utilize either naturally occurring or fermentation acids, forming carbonates with calcium salts and increasing the pH.
5. Bacteria which attack the casein of milk, breaking it down (*peptonization*) to such an extent that the milk becomes watery in appearance and at the same time producing obnoxious odors or flavors in the milk.

The five types of metabolic changes are found in milk in varying degree. The preponderance of a particular group of bacteria and the temperature of storage usually determine the manner in which the milk will spoil. In addition to the changes listed above, some species of bacteria produce both acid and gas, and some species develop a reducing intensity sufficient to reduce the oxidation-reduction indicator (litmus or methylene blue) to its colorless form (leuco base). These reactions are frequently of value in the identification of pure species of bacteria inoculated in sterile milk. Certain of these changes are also employed in the preparation of various milk products, fermented milk, cheese, and butter.

### Fermented Milk

Milk is an important article of the diet for many pastoral people, but because of the rapidity with which it spoils, the milk is usually fermented before it is consumed. In the fermented condition it can be kept for a longer period of time. Fermented milk is considered a delicacy by some peoples, and its consumption is also widespread because of supposed therapeutic value. Metchnikoff concluded that longevity in Bulgaria was due to the consumption of large quantities of milk soured by the action of *Lactobacillus bulgaricus*. This organism, introduced in sour milk, was supposed to develop in large numbers in the intestinal tract and to prevent the growth of proteolytic bacteria because of the large quantity of lactic acid produced. This decreased proteolytic activity of bacteria led to a decrease in so-called autointoxication due to the absorption of putrefactive wastes by the individual and hence greater chance of survival. *L. bulgaricus* does not become established readily in the digestive tract of man, and there is little evidence that its establishment would actually be conducive to long life. A related organism, *L. acidophilus*, is a common inhabitant of the intestinal tract but is nor-

mally outgrown by *Escherichia coli* and other species more suited for growth in either a saccharine or proteinaceous environment.

Many fermented milk products involve the action of more than one type of organism. Various products such as yoghurt, matzoon, leban, kumiss, or kefir result from the action of lactic acid bacteria (either rods or cocci or both) followed by alcoholic fermentation induced by yeasts.

### Cheese

There are some 20 types of cheese and over 400 varieties prepared from the milk of different animals and under different conditions to elicit the characteristics desired in the final product. The term cheese denotes a product made from the curd obtained on coagulation of the casein of whole milk, skimmed milk, or milk enriched with cream. Curd formation is induced either by the action of lactic acid bacteria or by the enzyme system rennet (commonly obtained from calves' stomachs), or by a combination of the two. The curd so formed is then modified by special treatments to produce the desired cheese. Cheese consists mainly of the partial decomposition products of casein together with fat, salts, sugar, and other constituents of milk.

Cottage cheese, which is actually more a form of sour milk than of cheese, is an example of the acid-type-curd variety of cheese. It is made commercially from pasteurized milk inoculated with a pure culture of *Streptococcus lactis*. This bacterium develops quite rapidly in the milk, lactose being fermented with the production of sufficient lactic acid to induce curdling. At the same time other enzymes of the bacteria, and to a slight extent of the milk itself, elicit changes in the milk which impart the characteristic flavor to the curd. The curd is separated from the whey, excess water drained off, and the cheese so obtained is salted, often mixed with some cream, and is then ready for use. Cottage cheese is not ripened by continued microbial action such as is involved in the preparation of most cheeses.

Most types of cheese are produced from the curd obtained when milk is coagulated with rennet, generally after growth of lactic acid bacteria has occurred to an extent sufficient to develop the desired acidity. The rennet-curd cheeses can be divided into two main groups, soft cheese and hard cheese. A hard cheese results when the curd is separated from the whey by the application of pressure sufficient to remove most of the free water. This gives a hard, tough curd which requires considerable time to ripen and which does not soften to an appreciable extent during the ripening process. Soft cheeses, on the other hand, are prepared from a curd obtained by allowing the whey to drain from the curd without the application of pressure. Ripening occurs in the presence of considerable



moisture, and a much softer product is produced. Cheddar, Cheshire, Edam, and Swiss cheeses are typical examples of hard cheese while the soft cheeses are represented by types such as Roquefort, Camembert, and Limburger.

A Cheddar type of cheese is the one produced to the greatest extent in the United States. Cultures of lactic acid bacteria are added to pasteurized milk, and rennet is added after the milk has "ripened" sufficiently to develop a slight acidity. The curdling enzyme, rennin, in rennet rapidly converts the milk into a firm, jelly-like mass, which is cut with special knives into small, uniform pieces. The cut curd is heated slowly, the temperature being quite high for Swiss cheese but less so for Cheddar, and this causes the curd particles to contract. This curd is salted and placed in molds lined with cheesecloth through which the whey can drain. In a few days the curd particles are matted together, and the mold can be removed. During this time *Streptococcus lactis* predominates, but as the lactose diminishes, other lactic acid bacteria become more prominent. They continue to act within the cheese during the ripening period, which may require from several months to two years. The growth of molds is prevented within the cheese by the anaerobic conditions which develop and on the surface by a layer of paraffin or other material. During the ripening process the casein is broken down by the action of bacterial enzymes and also by the pepsin which was present in the rennet. The casein is converted into soluble compounds with a resultant increase in soluble nitrogen content of the cheese, and the digestibility of the material is greatly increased at the same time. Flavor and aroma produced during ripening are probably due to fatty acids, alcohols, esters, and neutral volatile products produced by the action of bacteria, or their enzymes, acting upon various constituents of the cheese. Undesirable changes could be induced by coliform bacteria, but the majority of these are killed during the pasteurization of the milk, and any survivors are generally checked by the lactic acid in the cheese. At times other bacteria capable of utilizing lactate may be present and cause undesirable changes. Intact Cheddar cheeses keep well over a period of time but are subject to spoilage by molds once the cheese is cut. Processed Cheddar cheese, i.e., cheese that is pasteurized after curing and blending, has even better keeping qualities but loses somewhat in flavor and aroma.

Swiss cheese is produced under conditions somewhat different from those in the production of the Cheddar type. Milk, as fresh as possible, is inoculated with rennet which has been previously incubated in whey cultures of lactic acid bacteria. Curd formation occurs in the presence of relatively large numbers of bacteria but not under particularly acidic conditions. The curd is cut into very small pieces and is heated at a

higher temperature than with Cheddar curds in order to produce an even drier cheese. The curd is then pressed and salted on the outside only. Heating kills some of the acid producers, and a bacterial flora rather different from that in Cheddar cheese develops. *Lactobacilli* may predominate at first, but large numbers of propionic acid bacteria such as *Propionibacterium freudenreichii* and *P. shermanii* develop during the ripening process. The latter species can ferment either lactose or lactic acid while the former ferments lactic acid only, both with the formation of acetic and propionic acids and carbon dioxide. The production of this gas is responsible for the development of the characteristic holes, or eyes, in Swiss cheese.

Soft cheeses like Limburger and Camembert are made from rennet curds, but they contain more water and less salt than the hard cheeses. They are molded into much smaller units than the hard cheeses in order to promote diffusion, since the soft ones are ripened primarily by enzymes which diffuse into the cheese from microorganisms growing on the surface. Cheese of the Limburger type is produced by keeping the surface of the cheese moist to stimulate the growth of bacteria. Proteolytic enzymes are produced, diffuse into the cheese, and gradually digest the casein. Proteolytic digestion, actually putrefaction, is allowed to proceed to a greater extent than in other types of cheeses.

The Camembert type is produced like Limburger cheese except that the milk is inoculated with spores of *Penicillium camemberti*, and the surface of the cheese is kept dry to promote growth of this mold and of the yeast *Oidium lactis*. These two organisms are responsible for the main characteristics of this type of cheese.

Roquefort-type cheese has characteristics of both hard and soft cheeses. It is primarily a soft cheese, but the curd is ripened by the action of *Penicillium roqueforti* growing through the cheese. Aerobic conditions are obtained by piercing the cheese in many places with needles, the mold growing in these air channels and its enzymes utilizing both casein and fat to give the characteristic flavor of Roquefort cheese. The greenish mottled appearance of the cheese is due to the large numbers of *P. roqueforti* spores in the cheese.

It is apparent that cheese production is dependent upon the activity of a variety of microorganisms, conditions being selected which are most suitable for the growth of the desired types and the production of a particular cheese. In many instances the predominant species, and the conditions under which it develops, are sufficient to inhibit the growth of other species which would produce undesirable changes. Pasteurization of milk and the use of pure cultures, together with modern sanitary procedures, have greatly reduced spoilage and also transmission of patho-

genic species in cheese. Pathogens do not find suitable conditions for growth in cheese, and it is not so important a vehicle as milk and butter for the transmission of pathogenic forms which gain entrance to it.

### Bacteria in Milk

Milk as secreted by the healthy cow is sterile, but bacteria gain entrance through the teats into the udder, and milk drawn even under the most favorable conditions always contains bacteria. It may contain very few, or the number may run into the thousands per milliliter. These bacteria are generally cocci and are not pathogenic species. The numbers of bacteria in milk from diseased cattle may be much greater, and certain bacteria pathogenic for cattle may also be pathogenic for man. The most important bacteria pathogenic for both man and cattle are the streptococci causing septic sore throat, the tubercle bacillus, and the *Brucella* of undulant fever.

More microorganisms are introduced during the milking procedure, gaining entrance from the exterior surfaces of the cow, from the surroundings, and from the milker. Cleanliness greatly reduces the numbers gaining entrance from these external sources. Simple washing and wiping of the udder and of the hands of the milker can reduce the bacterial count to a tenth or less of that observed when this simple procedure is omitted. Cleanliness in the dairy barn is highly important, but cleanliness of the milking utensils, strainers, separators, and storage vessels is equally important. Mere washing of containers is not enough, since bacteria are held in tiny cracks and crevices, and the utensils should, therefore, be sterilized. Such a procedure when routinely employed can reduce the bacterial count as much as 75 per cent. Since bacteria are normally present in milk and since it is impossible to prevent the entrance of a few during milking and handling, it is essential to cool the milk as soon as possible and to keep it cold. Storage at a low temperature will inhibit the growth of all species commonly present in milk. Pasteurization will reduce the viable count by 95 to 99 per cent, and this product in sterile containers will remain fresh for a considerable length of time under refrigeration. The consumer, however, must be as careful in handling the milk as was the producer and distributor of a good grade of milk.

### Grading of Milk

Milk is graded primarily on the basis of the bacterial count. The total bacterial count is determined by counting the organisms in stained smears of a definite volume of milk spread over a known area of a microscope slide and the viable count by the plate method, suitable dilutions being plated out. Another method, the reductase test, serves as a rapid and



easy method of determining the approximate numbers of bacteria in samples of milk. Bacteria in milk reduce methylene blue to the colorless leuco base under anaerobic conditions, the methylene blue acting as a hydrogen acceptor. The time required for reduction is approximately inversely proportional to the numbers of bacteria present; the greater the number, the less the time required for decolorization. The test is reported in terms of the time required for decolorization of 1 ml. of 1:20,000 methylene blue by 10 ml. of milk at 37°C., and is interpreted as follows:

Class 1. Excellent milk, not decolorized in 8 hr.

Class 2. Good milk, decolorized in less than 8 but not less than 6 hr.

Class 3. Fair milk, decolorized in less than 6 but not less than 2 hr.

Class 4. Poor milk, decolorized in less than 2 hr.

In a similar reductase test the dye resazurin is substituted for methylene blue, the advantages being that resazurin is reduced more rapidly than methylene blue. The test is particularly valuable in detecting mastitis, an infection of the udder. No reduction of the dye in 1 hr. indicates an excellent milk as regards bacterial content, complete reduction within an hour a very poor quality, and intermediate degrees of reduction indicate intermediate qualities. Specific counts for coliform bacteria have also been suggested, but their value is still debatable.

Standards for milk vary somewhat in different localities, but in many states or municipalities they are based on those established by the U.S. Public Health Service Milk Ordinance (1939). The highest grade of milk is certified milk, a product closely safeguarded at every step during its production, collection, and distribution according to rules established by medical milk commissions. Certified milk is collected in sterilized containers and under clean conditions in the dairy from cattle which are frequently examined for tuberculosis and other diseases. The milkers are also subject to frequent health examinations. Milk collected under less carefully controlled conditions is graded as A, B, or C. The suggested standards for milk can be summarized as follows:

**Raw Milk.** *Certified.* This must conform to standards set up by the American Association of Medical Milk Commissions, the usual standard being a count not in excess of 10,000 bacteria per milliliter. All milk having a count in excess of this number is placed in one of the following grades:

*Grade A.* The average bacterial plate count must not exceed 50,000 per milliliter, or the direct microscopic count must not exceed 50,000 per milliliter if clumps are counted or 200,000 if individual bacteria are counted, or the average reduction time of methylene blue must not be less than 8 hr. If Grade A milk is to be pasteurized (which should be



done), the permissible counts before pasteurization are 200,000, 200,000, and 800,000 per milliliter, respectively, and a decolorization time of not less than 6 hr.

*Grade B.* Raw milk is classified as Grade B if it has a higher bacterial count than that permissible for Grade A (or from cattle that are not known to be free from contagious abortion) but not exceeding 1,000,000 per milliliter in the plate or total clump counts or 4,000,000 individual cells per milliliter. The average reduction time should exceed 3.5 hr.

*Grade C.* Raw milk which violates any of the standards for Grade B raw milk is classified as Grade C.

**Pasteurized Milk.** *Certified Milk, Pasteurized.* This is certified raw milk which has been pasteurized under conditions desirable for the pasteurization of Grade A milk. (Certified milk need not be pasteurized, but it is a worth-while safeguard.)

*Grade A, Pasteurized.* This is Grade A raw milk pasteurized, cooled, and bottled in the milk plant. Plate count must not exceed 30,000 per milliliter after pasteurization and before delivery.

*Grade B, Pasteurized.* This is pasteurized milk from raw milk of Grade B quality. Bacterial plate count after pasteurization and before delivery must not be in excess of 50,000 per milliliter.

*Grade C, Pasteurized.* This is pasteurized milk of less than Grade B quality.

A high bacterial count in milk does not mean that the milk is unsafe, i.e., carries bacteria pathogenic for man, since all the organisms could be saprophytic species. Infections have been traced to milk having a very low bacterial content. All that the count indicates is that the milk has come from diseased cattle, has been collected or handled under unclean conditions, or that the milk has stood for some time in a warm place. The first two conditions indicate a *possibility* for the entrance of pathogenic forms, the latter condition greater chance for their growth if present.

### Ecology of Milk

Freshly drawn milk contains a substance or substances capable of exerting a bactericidal effect against coliform and possibly other species of bacteria. This bactericidal action varies with milk from different cows, with the bacteria present in the milk, and the temperature of incubation. It is destroyed by heating the milk at 53°C. for 30 min. The length of the bactericidal phase varies, and there is no adequate explanation of its existence and why bacteria do develop after a time, unless the material is extremely labile. One suggestion is that it is a protective substance for the suckling calf.

It is common experience that raw milk standing in a relatively cool place tends to sour. Normally milk is delivered in a tall bottle with

little surface exposed to the air, thus reducing the ease with which oxygen can penetrate into the milk. This diffusion of oxygen is further hindered by the layer of cream, which rises to the surface and forms an oil seal. Bacteria in the milk soon utilize the dissolved oxygen, and anaerobic conditions develop, thus favoring the growth of lactose-fermenting organisms. Coliform bacteria tend to predominate if the milk is stored in a warm place, while lactic acid bacteria generally gain the ascendancy when the milk is kept cool. *Streptococcus lactis* apparently is best adapted for growth in milk, as it generally grows more rapidly and to a greater extent than any of the other lactic acid bacteria in milk. It can develop over a relatively wide temperature range and at acidities as low as pH 4. It is a saprophyte not present in milk in the udder, but it almost invariably gets into milk during the milking process, apparently from plant material, dust, and from the exterior surfaces and possibly feces of the cow. After milk has undergone preliminary souring by *S. lactis*, various species of lactobacilli may continue to develop as they tolerate somewhat greater acidities than *S. lactis*. Milk soured by the combined activities of the lactic acid streptococci and bacilli is no longer a suitable medium for the growth of most bacterial species, but yeasts and molds can develop on the lactic acid or the proteinaceous material in the sour milk, particularly if there is access to oxygen.

The smaller the initial count and the lower the temperature, the greater the time required for the milk to sour or to undergo "abnormal" fermentation (changes other than the "normal" type of milk fermentation). The abnormal fermentations include such changes as color production by chromogenic bacteria; sweet curdling by species which secrete rennin-like enzymes; ropiness, caused by capsulated bacteria which form prominent capsules or large amounts of gummy material; gaseous fermentation by coliform bacteria or by yeasts; and proteolytic fermentations. In some instances one species or a group of closely related species will gain and maintain ascendancy; in other instances there will be shifts with time (actually with changes in the environment) in the predominant species. In general, however, the bacterial flora of samples of the same milk stored at different temperatures will be as follows:

0 to 10°C. Psychrophilic fluorescent bacteria develop slowly but may produce an enormous population in time. Either saccharolytic or proteolytic species of *Pseudomonas* tend to predominate, at times causing discoloration. Lipolytic action may be evident in some instances.

10 to 20°C. Psychrophilic fluorescent bacteria as above together with different species of *Mycobacterium* and *Alcaligenes*, the latter tending to cause ropiness. *Streptococcus lactis* frequently is the early predominant species at the upper part of this temperature range.

20 to 40°C. *Streptococcus lactis* and other lactic acid streptococci at lower temperatures in this range, with lactic acid bacilli (*Lactobacillus*) developing more

readily at the higher temperatures. *Proteus vulgaris* may at times predominate and elicit peptonization, or coliform bacteria may bring about a gaseous fermentation. Actinomycetes may also develop and produce a musty odor and an obnoxious taste.

40 to 60°C. Lactic acid bacilli in the lower part of this temperature range, various thermophilic species in the higher range. Some samples may show no growth of bacteria at 60°

### Pasteurization

Sanitarians realize that milk, being an excellent culture medium for many pathogenic bacteria, is the most important food-stuff in the transmission to the consumer of infectious diseases such as tuberculosis, undulant fever, and streptococcal infections from the cow, and diphtheria, typhoid and paratyphoid fevers, and bacillary dysentery from milk handlers. Pasteurization, either the holding of milk at 62°C. (143°F.) for 30 min. or "flash" heating with the milk being held at 71°C. (160°F.) for 15 sec., is the most important factor in the provision of a safe milk supply. All common pathogenic species of bacteria will be killed during either holding or flash pasteurization, and if the milk is immediately cooled and bottled in sanitary containers, it will remain free of pathogens, the taste will not be altered appreciably, and the vitamin content will remain at

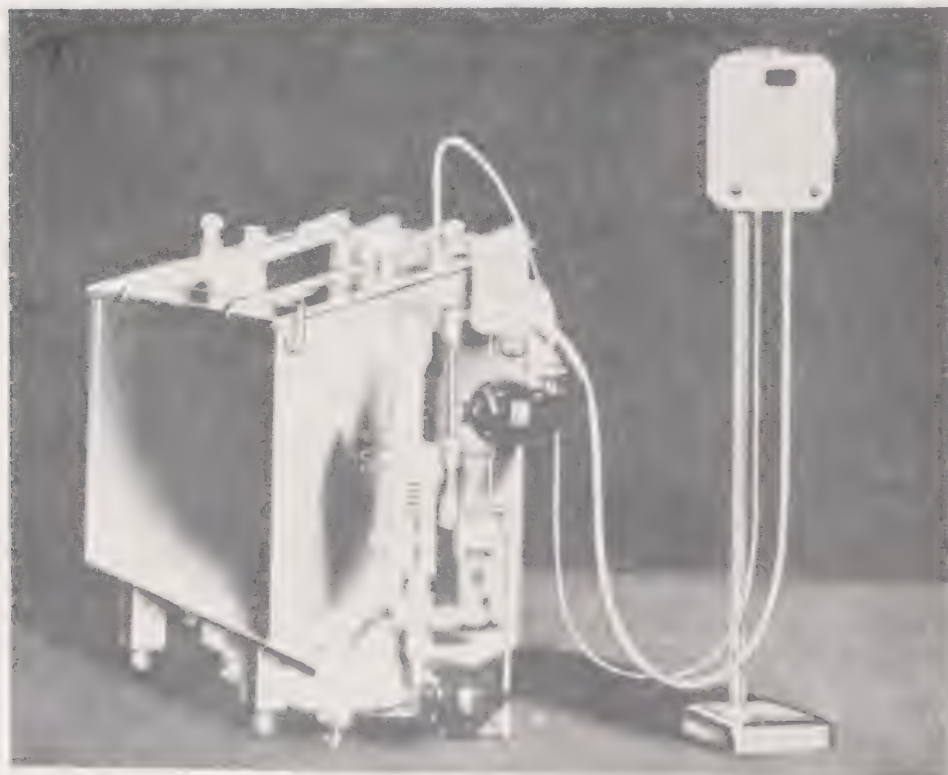


FIG. 18-1. Photograph of a milk-pasteurizing machine equipped with an automatic temperature control and temperature recorder. (Courtesy of the Cherry-Burrell Co.)



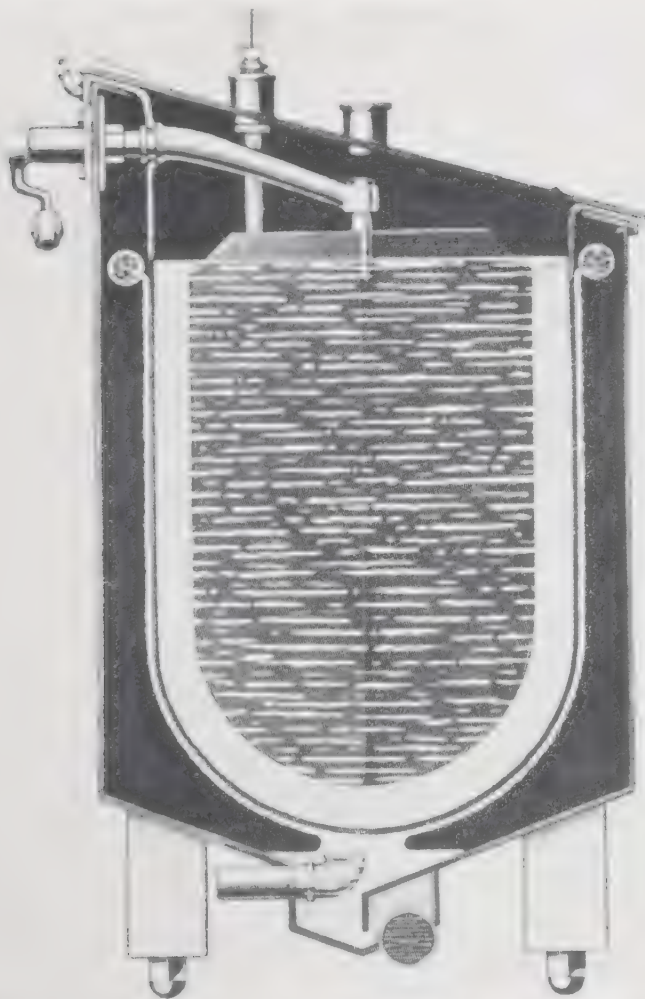


FIG. 18-2 Cross section of the pasteurizer illustrated in Fig. 18-1, showing flow of hot water around the wall of the milk tank and the paddle to provide agitation of the milk to assist in the transfer of heat. (Courtesy of the Cherry-Burrell Co.)

a level near that of the raw milk. Pasteurization is not a cure-all, however, since it will not remove bad tastes or odors in dirty milk or from milk in which large numbers of bacteria had developed before pasteurization. It is simply a process for the destruction of pathogenic and other heat-labile species which might be present in the milk before pasteurization, without causing appreciable change in the quality of the milk from that which it possessed at the time of pasteurization.

An enzymatic reaction, the *phosphatase test*, is available for rapid determination of the efficiency of pasteurization. The enzyme phosphatase is always present in raw milk, and this enzyme liberates phenol from its phosphoric ester, the phenol reacting with added 2,6-dibromoquinone-



chloroimide to develop a blue color. Phosphatase is heat-labile, and 96 per cent is inactivated at 143 F. in 30 min., 100 per cent inactivation occurring in the same time at 145 F. If no color develops during the phosphatase test, it indicates that pasteurization has been sufficient to

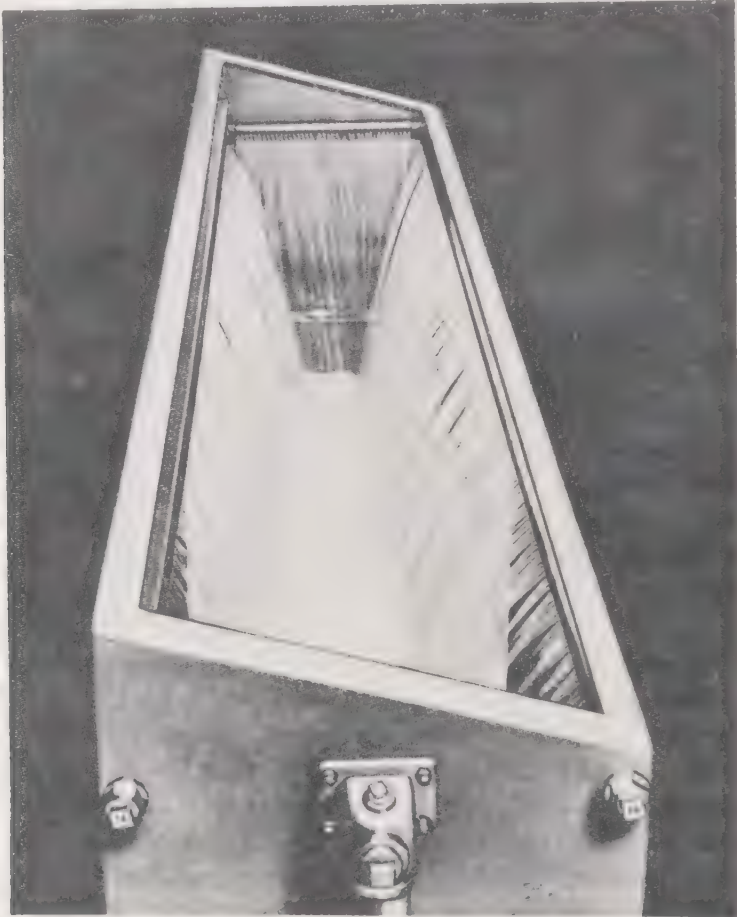


FIG. 18-3 Milk tank removed from the pasteurizing machine to show the flow of heating water around the tank during pasteurization. (Courtesy of the Cherry-Burrell Co.)

destroy all pathogenic species and that raw milk has not been added to the pasteurized milk after pasteurization. Underheating by 1 degree or contamination with as little as 0.1 per cent raw milk can be detected by the phosphatase test, which serves as an efficient check on the honesty of the operator or the efficiency of his equipment. Direct microscope counts would have no value as a check on pasteurization, and plate counts, while of value as checks, require time for colony growth to become apparent, and during that time much of the milk could have been consumed.

### Butter

Butter is made from either sweet or sour cream and, when prepared from the latter, has a taste and aroma due to the action of several species of bacteria. Since pathogenic bacteria such as *Mycobacterium tuberculosis* or *Salmonella typhosa* are present at times in milk, it is highly desirable to pasteurize the milk or cream before it is employed in the preparation of butter. Pasteurization also serves to kill other extraneous bacteria which might cause butter to spoil on standing. In the manufacture of sour-cream butter the pasteurized cream is inoculated with a butter starter, a mixture generally containing *Streptococcus lactis*, *S. cremoris*, *Leuconostoc citrovorum*, and *L. dextransicum*. The streptococci cause the cream to sour while the latter organisms produce primarily acetic acid, acetylmethylcarbinol, and diacetyl, which are responsible to a great extent for the characteristic taste and aroma of sour-cream butter. After the cream has soured, it is then churned, and the butterfat which separates is washed, salted, and worked into the desired shape. The final product contains approximately 82 per cent fat, 14 per cent water, 2.5 per cent salt, and traces of casein, lactose, and other substances. The water with its dissolved solutes is dispersed in small droplets in the fat, and since the salt concentration in the water is quite high, most species of bacteria are inhibited and the butter tends to keep well. Halophilic organisms if present (frequently introduced as contaminants in the salt) may grow and cause spoilage, butyric acid fermentation frequently being noted. Species of fat-splitting bacteria, generally of the genus *Pseudomonas*, are commonly encountered and in addition to the fatty acids responsible for rancidity may also produce substances, such as trimethylamine, which impart a fishy odor to butter. Growth of molds is generally inhibited by anaerobic conditions within the butter and by the common practice of tightly wrapping it to inhibit surface aeration and oxidations.

### Miscellaneous Products

Milk products such as evaporated or condensed milk, milk powder, or ice cream are not products of microbial activity in milk, but they may serve as vehicles for the transport of bacteria. Evaporated or condensed milks contain less than one-half the water content of milk itself. The former product is usually sterilized, while the latter is preserved by the addition of about 40 per cent sugar. When prepared from pasteurized milk, neither is apt to be involved in the transmission of pathogenic bacteria unless they are diluted and allowed to stand under unhygienic conditions. The same statement holds true for milk powders or ice cream.

**BACTERIA IN OTHER FOODS**

In a few instances, foods can be preserved as a result of microbial activity, while in the vast majority of cases, preservation is possible only if the foodstuff is kept free from microorganisms or if their growth and activity are inhibited. The main examples of the foods (other than cheese) preserved by microbial action are sauerkraut, ensilage, and pickles, and possibly the aged eggs of the Chinese.

**Sauerkraut.** Cabbage and most other vegetables contain between 3 and 5 per cent sugar, which can serve as food for bacteria once the plant cells are disrupted. In the manufacture of sauerkraut the cabbage is finely shredded, a small amount of salt is added, and the mass is firmly packed in a deep container. The salt draws water out of the cells, and this aids in their final disruption. A considerable amount of nutrient material becomes available for the bacteria initially present on the cabbage, and growth is soon initiated. Anaerobic conditions are rapidly established in the bulk of the cabbage, and then lactic and propionic acid bacteria generally gain numerical superiority over other species. The cabbage definitely becomes sour, and the high degree of acidity developed, together with depletion of readily available nutrients, inhibits further microbial decomposition except on the surface. Here there is access to air, and molds or oxidative scum yeasts can develop at the expense of lactic acid. Putrefaction can set in once the lactic acid has been oxidized. These changes can be prevented by keeping the sauerkraut well submerged or by canning it when fermentation is complete. Pure cultures of bacteria are not employed in making kraut, the desired species practically always being present on the cabbage and being able to crowd out other species of bacteria under the conditions prevailing in the kraut fermentation tanks.

The same principle is employed on the farm for the preservation of food for animals. Shredded cornstalks and leaves, grass, or other forage crops are packed in large cylindrical containers called silos. The packed shredded material undergoes the same physical and microbial types of change as cabbage in sauerkraut production. The fermented materials, known as ensilage, or silage, keep well under anaerobic conditions and serve as an excellent food for cattle and other animals during the winter months. In rare instances, anaerobes such as *Clostridium botulinum* may develop and cause silage poisoning.

**Pickles.** Microbiologically the preparation of pickles is essentially similar to the production of sauerkraut. In this industry, however, the material to be fermented is not shredded. Dill pickles are prepared by the fermentation of cucumbers in 5 per cent salt solution, certain desired tastes being obtained by addition to the pickles of dill seeds—or parts

of the dill plant itself—and leaves of other plants such as grapes. Some sugar and other nutrients pass into solution from the cucumbers, and aerobic growth is initiated but soon stops with the development of anaerobic conditions. Lactic acid bacteria and similar anaerobic species outgrow other bacteria, and the characteristic flavors develop during the course of the lactic acid fermentation. Pickles will keep almost indefinitely if access of air is prevented.

Brine pickles are prepared by fermenting cucumbers in 10 to 15 per cent brine, a concentration of salt sufficient to inhibit most species of bacteria. A lactic acid fermentation does, however, occur very slowly, and the end result is a pickle similar to the dill variety. The excess of salt can be leached out of brine pickles, and they can then be converted into sweet, sour, or American dill pickles by appropriate treatment with spices, vinegar, sugar, or other agents. Green-tomato, cauliflower, onion, pepper, or green-bean pickles are made in the same manner. String or snap beans are sometimes preserved by a process similar to that employed for brine pickles and are used directly as a vegetable after washing free from salt.

### NONMICROBIC PRESERVATION OF FOOD

Cabbage can be preserved in the form of sauerkraut and certain vegetables or fruits as pickles, but all these food products have a sour taste, which is not desirable in most foods. It is therefore necessary to employ other methods for the preservation of foods from a time of abundance to one of scarcity. The aim of all methods is the inhibition or destruction of all organisms present in or on the food to be preserved. Preservation can be accomplished in a variety of ways, the method of choice generally depending upon the type of food to be kept. Meat, for example, being rich in proteins is subject to proteolytic decomposition, is more subject to contamination with animal pathogens than are vegetables, and makes a better medium for the growth of pathogenic species. Cooking under the conditions necessary for preservation by canning so alters its flavor that meat is not in general preserved in this manner. The inspection of animals and their carcasses by competent inspectors, cleanliness in the slaughterhouse, and cold storage serve as means of supplying meat safe for human consumption. Vegetables and fruits, while they can be preserved by quick-freezing and cold storage, are at present preserved to the greatest extent in cooked forms in sealed containers. Other methods of preservation have limited use but are desirable for specific reasons. The various methods commonly employed in the food-preservation industries can be summarized as follows:



## 1. Inhibition of microbial action by:

- a. Storage at temperatures below 0°C—vegetables, fruits, meats, eggs, and ice cream
- b. Refrigeration—vegetables and fruits or their juices, meats, eggs, and dairy products
- c. Dehydration—fruits, vegetables, eggs, milk, and meats
- d. Osmotic-pressure effects—high sugar content in jellies, jams, and condensed milk, and salting of meats and vegetables
- e. Specific chemicals—food preservatives such as sodium benzoate (catsup), acetic and lactic acids, or spices and essential oils

## 2. Destruction of microorganisms by:

- a. Pasteurization—milk, fruit juices, and mild alcoholic beverages
- b. Boiling—most fruits and acidic vegetables
- c. Heating under pressure—vegetables, fruits, and meat products

There are two major aims in the food-preservation industries: the maintenance of a product with the desired characteristics and the prevention of the spread of infectious agents by contaminated food. Pathogenic microorganisms present in foodstuffs preserved by the inhibition of microbial action may remain viable over considerable periods of time and may subsequently multiply if the foodstuff is allowed to stand under conditions favorable for growth of the pathogenic forms. The spread of infectious agents can be practically eliminated by the selection of foods not contaminated with species pathogenic for man, by processing them under sanitary conditions, and by periodic examinations of food handlers to determine that they are not carriers of pathogenic organisms, particularly those of the enteric group.

**Inhibition of Microbial Action.** The interior tissues of the plants and animals we use for food are generally free from microorganisms as long as the tissues are alive and healthy. The living cell, as has been previously considered, requires the continuous provision of energy if it is to be maintained. Once the edible portions of plants or animals have been harvested, an external source of energy is no longer available to the component cells, and they begin to disintegrate. This spontaneous breakdown of the cell is frequently aided by the enzymes present in the cell and is spoken of as autolysis. Autooxidation of cellular material also occurs, and the foodstuff begins to spoil, i.e., change in odor, taste, or appearance. Such change paves the way for invasion by microorganisms which greatly accelerate decomposition.

Enzymic action, either of the tissues or of the contaminating microorganism, is greatly retarded or completely blocked at low or high temperatures, in the presence of very little moisture, or in the presence of inhibitory chemicals. Low-temperature inhibition of enzymic action is being employed to an increasing extent at the present time, particularly with the modern development of methods of quick-freezing of fruits, vege-

tables and meats. When foodstuffs are quick-frozen, less change occurs in their appearance, flavor, and palatability than in any other method of preservation. Many bacteria, however, remain viable in the quick-frozen state for months or even years, one explanation being that the ice crystals formed in this process are so small that the cell is not damaged and remains in a state of suspended animation. When quick-frozen foods are held at or near  $-18^{\circ}\text{C}$ . ( $0^{\circ}\text{F}$ .) or at slightly lower temperatures, they can be preserved over long periods of time. When thawed and used immediately, there is no more danger of the spread of infectious agents than there was with the original material, providing that it has been handled in such a manner as to prevent contamination with pathogens. Simple refrigeration or cooling is not enough to check entirely the action of enzymes, and foods do spoil spontaneously or as a result of microbial action in the refrigerator, cooling simply acting as an enzymostatic agent.

The same general principle, inhibition of enzymic action, applies to the preservation of food by dehydration, high osmotic pressure, or the use of inhibitory chemicals. Dehydrated foods keep well and occupy much less storage or shipping space than does the fresh material, but there are two important disadvantages in many instances: the change in appearance and other properties which accompanies dehydration and the difficulty of getting water back into the dehydrated material before it is consumed. Dehydration is employed to a considerable extent in the preservation of certain types of food and is one of the oldest methods commonly employed by man, particularly for the preservation of meat. Large numbers of bacteria, yeasts, or molds may be present in dehydrated material, but they do no harm and many die on standing. Osmotic-pressure effects, generally produced by sodium chloride or by sugar, are in many respects a form of dehydration, the salt or sugar drawing water from the plant or animal cells.

We have considered the preservation of certain foodstuffs by means of the inhibitory acidity developed by fermentation. Preservation in a similar manner can be accomplished by the addition of specific acids, generally acetic (vinegar) or lactic, to the food, but this method is limited to those foods which man relishes with a sour taste. A number of spices contain essential oils or other components with weak bacteriostatic properties, and they are used to a limited extent in food preservation. Taste, however, limits the concentrations which can be employed, and spices have little general value as preservatives. Smoke, also, acts in a similar manner, but at best certain of its constituents are only mild bacteriostatic agents, and it is usually employed in conjunction with salting, heating, or drying. Chemical agents such as boric, salicylic, or benzoic acids were formerly employed to a considerable extent as preservatives, but their

value, in the concentrations which can be used, is very doubtful. Stronger enzymatic or cellular poisons cannot be employed, as they would be toxic to man as well as microbe.

Antibiotics have been tested regarding their value for use as food preservatives, and they show some promise for this purpose. In particular they appear to be of potential value in the preservation of meat. It has been reported that Aureomycin (chlortetracycline) injected into beef carcasses inhibits or prevents spoilage for many days at temperatures which lead to rapid spoilage of the untreated meat. The treated meat at the same time tends to be more tender than the untreated portion. Fish and poultry will also keep for longer periods if the dressed material is cooled in ice containing the antibiotic. The amounts of the antibiotic required are relatively small and most or all would be removed or destroyed during preparation for the table. More work is needed along these lines before the value of the procedure is established and it is employed on a large scale. The value of radiations for the sterilization of food is also under investigation.

Many of our foods are heated before they are consumed, and we are used to the cooked taste. Heating to a sufficiently high temperature destroys enzymes and the cells from which they were derived. Once all cells have been killed, the food will keep indefinitely provided that it is not exposed to contamination or to sufficient oxygen to elicit marked oxidative change. Preservation of foods heated in closed containers was introduced by Appert in France in 1807, but spoilage did frequently follow attempted preservation by the original crude methods of the infant canning industry. Heating by boiling was not sufficient to destroy spores which could develop later in a nonacidic food, and the methods of sealing were not always satisfactory. Preservation by boiling is still employed to a limited extent, particularly in the open-kettle and water-bath procedures in the home, but is satisfactory only with foods which are acidic in character. Meats, corn, beans, and similar vegetables cannot in most instances be preserved by methods in which only the vegetative cells are killed.

The commercial packer and many housewives now heat the canned material under pressure in order to obtain a product which is sterile, or reasonably so. Too long a period of heating, or heating at too high a temperature, can so change the nature of the food as to render it objectionable, and it is probably safer to say that most canned foods are preserved by "processing" at temperatures above the boiling point rather than to say that they are preserved by sterilization. In many instances, sterilization is accomplished, but an occasional spore or highly resistant vegetative cell may remain viable and cause spoilage. Two main types of spoilage are recognized. *Swells* are cans in which fermentation with

production of gas has occurred to such an extent that the ends of the can are bulged. In relatively rare instances, swelling may be caused by chemical reactions in which hydrogen is ordinarily the gaseous product. *Flat soured* are cans in which fermentation with the production of acid but little or no gas has occurred. In some instances, spoilage is induced by *Clostridium botulinum*, which produces a highly potent toxin (see Chap. 23), and a drop of such spoiled material may contain sufficient poison to be lethal to man. Food showing any evidence of spoilage should be immediately discarded and in such a manner that animals cannot consume it. Furthermore, it should never be tasted first to "see if it is all right."

The canning industry is one of the major industries in the United States, the present annual output of canned products being in the neighborhood of 6 to 7 billion No. 2 cans. The canning industry has made careful determinations of the thermal death points and thermal death times of different bacteria and spores in different foods packed in various ways and in containers of different sizes. *Thermal death point* signifies that temperature at which all test organisms are killed within 10 min. under the conditions of the test, while *thermal death time* expresses the time required for destruction at a lethal temperature under specified conditions. These values, particularly that of thermal death time, serve as a guide along with practical experience and the necessity of producing a palatable product. The nature of the canned material and the size of the container must also be considered on the basis of ability of heat to penetrate throughout the contents. The application of scientific principles has been responsible for the development to an advanced state of the modern canning industry, an industry predicated upon the necessity of preventing microbial action rather than upon employing it for the production of desired products.



## CHAPTER 19

# INDUSTRIAL MICROBIOLOGY

In the broadest sense industrial microbiology includes all applications of microorganisms to the preparation of materials of commercial value. The production of cheese, butter, sauerkraut, and other substances considered in the preceding chapter is actually a part of industrial microbiology. It, however, was considered along with food preservation since the changes induced by the various microorganisms involved resulted in products with better keeping qualities than the starting food-stuff. In this chapter attention will be focused primarily on microbes and microbial activity involved in the production of chemical entities. Such a division is an arbitrary one and is employed here for convenience only.

Most processes in industrial microbiology are highly technical in character and are carried out in economic competition between the various producing companies. This has led to the search for better organisms or strains thereof and for cheaper raw materials and to the determination of optimum environmental conditions, in addition to the development of improved equipment and recovery processes. Research in applied microbiology has increased our store of knowledge concerning microorganisms and their behavior in general. Individual species or strains have been found which are most suited for a particular process, and they are distributed amongst the yeasts, the molds, and the bacteria. Since the greatest single industrial application of microorganisms is in the production of alcohol, this process will be considered in most detail to illustrate fundamental principles involved in industrial applications of microbial activity.

**Industrial Alcohol.** Ethyl alcohol production in the United States has increased with increasing industrial demand from an annual output of approximately 10 million gal. in 1920 to 120 million gal. in 1940. This production was stepped up to over 500 million gal. annually during the war years. These figures do not include the alcohol in beer, wine, and other alcoholic beverages produced by fermentation but do serve to illustrate the magnitude of this industrial application of microbiology.

Alcohol is produced on a commercial scale by the fermentation of sugar under carefully controlled conditions with selected strains of *Saccharo-*

*myces cerevisiae*. Molasses served as the main source of sugar before the Second World War, but during that period it became necessary to substitute starchy grains or cellulose for sugar, which was in short supply. Starch, from grain or potatoes, or cellulose, has to be hydrolyzed by chemical or microbial action to sugar before it is available to yeast, which lacks diastase, the starch-splitting enzyme. This hydrolysis requires additional equipment and procedures and will be considered briefly under brewing.

Molasses is diluted with water to a sugar content of 10 to 12 per cent; the solution is acidified to a pH of 4 to 4.5; and ammonium salts and phosphates are added if necessary to supply sufficient nutrient matter for growth of the yeast. Pure cultures of yeast are maintained in the laboratory, and transfers are made from these cultures to larger and larger volumes of culture media for the preparation of the organisms employed in the inoculation of the material to be fermented. Fermentation is frequently carried out in vats of 250,000-gal. capacity, and for fermentation on this scale an inoculum of approximately 11,000 gal. of yeast culture is required. Aerobic conditions are maintained for a time in the fermentation vat to promote growth of yeast to an extent sufficient to provide enough cells for a rapid fermentation. The air supply is then shut off; anaerobic conditions become established; and fermentation is generally complete within 2 to 3 days, the alcohol content of the fermentation liquor being around 10 per cent. Since the fermentation is carried out at a relatively low pH, the growth of most species of bacteria is inhibited, while anaerobic conditions which prevail for most of the time are detrimental to the molds. Contamination, therefore, is a minor problem in comparison with some of the other fermentation industries.

The carbon dioxide of fermentation is collected and compressed into cylinders or converted into dry ice, a yield in the neighborhood of 60,000 lb. of carbon dioxide being obtained from a 250,000-gal. fermenter. The collection and utilization of various products reduces the cost of production of the alcohol itself. The fermented liquor is distilled with the production of 60 per cent alcohol, which on distillation in refining columns gives fusel oil and around 16,000 gal. of the 95 per cent grain alcohol of commerce. Potassium salts and carbon are additional by-products of molasses fermentation, while residues from the fermentation of grains or potatoes can be utilized as supplements for cattle food.

Since neither the fermentation nor the baker's strains of *Saccharomyces cerevisiae* form the starch-splitting enzyme diastase, starch must be hydrolyzed to fermentable sugar before it becomes available as a food for yeasts. Malt, prepared from sprouted barley grains, is rich in diastase and is commonly employed for the hydrolysis of starch to sugar. Molds produce considerable amounts of diastase, and they are used to a considerable extent for the production of this enzyme. The desired species,

generally *Aspergillus oryzae*, is cultivated on moist bran, the enzyme is extracted from the moldy bran and is added to the starch material to be hydrolyzed. In some instances the starch is hydrolyzed by the molds directly rather than by the enzyme separated from them.

**Glycerol.** Glycerol is ordinarily obtained from fats, but during the First World War the fat supply in Germany was extremely limited. It was known from Neuberg's studies that yeast fermented sugar in the presence of sulfites, or in an alkaline environment, with the production of glycerol. Sulfites bind acetaldehyde in such a manner that it is not available as a hydrogen acceptor in the normal scheme of fermentation, and dihydroxyacetone phosphate acts in its place, being reduced to glycerol. Under alkaline conditions the reduction of acetaldehyde is inhibited, and approximately one half of the sugar fermented is converted via dihydroxyacetone to glycerol, the other half being dissimilated with the formation of acetaldehyde, which undergoes a Cannizzaro reaction, one molecule being reduced to ethyl alcohol at the expense of a second molecule which is oxidized to acetic acid. Glycerol is difficult to separate from the fermentation mixture, and the process is of little economic importance normally. The process does, however, illustrate how the activities of microorganisms can be influenced by changes in the nature of the environment.

**Brewing.** Brewing, or the production of malt beverages, particularly beer, is an ancient industry, probably dating from early Egyptian times. The brewing process is similar to the production of industrial alcohol from grains in that the starch must be converted to sugar and subsequently fermented. The nature of the end product is such, however, that there are a number of steps peculiar to the production of beer.

The chief raw material for beer is barley, or other grains. The first step in beer production is the preparation of malt from barley, which is soaked in water and then permitted to germinate for about 10 days. Germination is then halted, and the "green malt" is next dried at 75 to 100° C., the temperature employed being determined by the type of malt desired, a light malt being obtained at the lower temperature. This dried malt contains a high percentage of starch, but during the germination process the starch-splitting enzyme, diastase, was produced and is present in the malt.

In the actual process (see Fig. 19-1, to which the figures in the text apply), malt and cereal adjuncts from storage bins (1) are mixed with hot water (2) in the cooker (3) to form a cereal mash. The mash is mixed with more malt and water in the mash tub (4), and during mashing the starch is hydrolyzed by the enzyme diastase to malt sugar, or maltose. When mashing is complete, the mixture is filtered (5), and the spent grains can be recovered for use in cattle food. The filtrate (malt extract, or



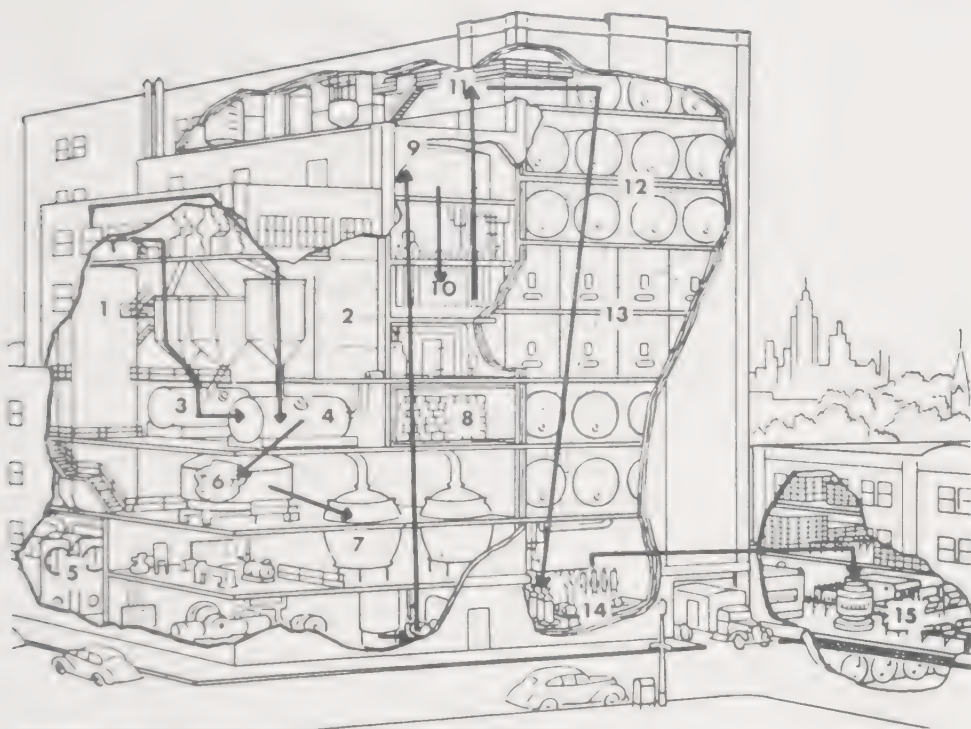


FIG. 19-1. Flow sheet for the production of beer. The numbers refer to descriptive material in the text. (Courtesy of the Armstrong Cork Co.)

wort) is next boiled with hops (7), which add desired flavor and at the same time an antiseptic oil somewhat inhibitory to lactic acid bacteria, which would find highly suitable conditions for growth in the wort. The hops are removed, and the hopped wort is pumped into the tank (9), flows through the cooler (10), and then into the storage tank (11). It is then inoculated (pitched) with a culture of selected yeast, and fermentation proceeds in the fermentation tanks (12). Top-fermenting yeasts are employed for the preparation of ale and bottom-fermenting strains for beer, strains of *S. cerevisiae* commonly being employed although *S. carlsbergensis* or *S. monacensis* is sometimes the organism of choice.

The fermentation is carried out at 6 to 12°C. for 8 to 10 days in the production of beer and for 5 to 7 days at 15 to 23°C. for ale. During this time most of the fermentable sugars are converted to ethyl alcohol and carbon dioxide together with smaller amounts of glycerol and/or acetic acid. Higher alcohols and acids are produced from amino acids, and these substances react to a limited extent, with the production of organic esters. The fermenting solution gradually becomes more acidic, and some of the protein and other complex organic substances such as resins settle out of solution.



When fermentation is complete, the beer passes into storage tanks (13), where it is aged at a low temperature. During aging, the suspended yeast cells and other materials settle out and the beer mellow or matures with the development of desired characteristics. After aging, beer is filtered, carbonated, and packaged in kegs (14), bottles (15) or cans, packaged beer being pasteurized at 60 to 61°C. for 20 min. before it is marketed. More than 70 million barrels (31 gal.) are produced annually by the action of somewhere in the neighborhood of 200 million lb. of yeast. This yeast, if it could be debittered economically, would have considerable use as a food adjunct.

Wines and hard cider are produced in a manner somewhat similar to the production of beer. Mashing is not required, since the fruit juices are rich in fermentable sugars, and hopping is not employed. The type of wine produced depends upon the nature of the grapes and the processing of the fermented liquid. Pure cultures of yeast are ordinarily not employed, sufficient yeasts normally being present on the fruits and gaining entrance to the juice expressed from them. The predominating species in vinous fermentation is *S. ellipsoideus*, which differs from beer yeasts in shape, in power to produce higher alcohols, and in the flavor it imparts to the fermented liquid. The addition of sulfurous acid to the juice greatly inhibits the growth of bacteria. The addition of pure cultures of the desired yeast would further ensure the development of the desired fermentation but is generally not necessary. Once fermentation is complete, enzymic changes may continue and aid in the ripening of the wine. Annual production is in the neighborhood of 150 million wine gallons, the bulk of the American production being in California wineries. Tartrates, which were formerly imported from Mediterranean countries, are becoming an important industrial by-product of wine making, recent developments indicating a possible annual production of 10 million lb.

The production of whiskies and other hard liquors is more closely related to industrial alcohol production from cereals. The fermented liquid is distilled and then aged for a considerable length of time in oak barrels. Many of the whiskies on the market at the present time are blends of aged whiskies and of alcohol. Annual production of distilled liquors is greater than 160 million tax gallons.

**Baker's Yeast.** The production of baker's yeast, selected strains of *Saccharomyces cerevisiae*, is an industrial operation of considerable extent, over 100,000 tons of yeast being produced annually in the United States. Here again the influence of environment on microorganisms is well illustrated. The distillers and brewers are interested in the conversion into alcohol of as much as possible of the carbohydrate employed, together with minimum yeast production necessary for the fermentation. The producers of baker's yeast, on the other hand, must convert sugar

into yeast substance with minimum production of metabolic waste products such as ethyl alcohol. In the fermentation industry metabolic products are desired, cells themselves in the yeast factories.

In early days it was observed that bread made from dough which had been allowed to stand for some time was frequently lighter in texture and of improved palatability. The mass of dough appeared to increase in size, to rise on standing, and this process could be facilitated by keeping a portion of the risen dough and adding it to subsequent batches. The use of such starters for the production of leavened bread became quite common but no one knew the cause of the phenomenon of dough rising, and frequently considerable trouble was encountered, the dough not behaving as it should and the bread produced from it having undesirable qualities. Frequently a sour-dough type of bread was produced, and, as we now know, this type of leavened bread results from the production of acid and gas by bacteria, generally *Acrobacter cloacae*, in the dough. Clostridia, which are capable of fermenting starch as well as sugar, begin to multiply when the dough stands for too long a period of time, and they produce butyric acid and other undesirable substances in the dough. Bacterial fermentations can generally be kept at a minimum by the use of large quantities of yeast and consequent decrease in time required for the dough to rise.

While compressed yeast for baking purposes was introduced by Mason in England in 1792, little was known about the nature of this product which was highly variable. With the realization that leavening is brought about by the action of yeast, it became possible to control more closely the leavening process as well as the production of starters for use in the baking industry or at home. The first marked advance in the preparation of baker's yeast was the development of the Vienna process in 1860. Kiln-dried malt and corn were ground together, mixed with water, mashed, inoculated with yeast, and the mixture allowed to ferment. About 10 to 14 per cent of the sugar was converted into yeast, considerable quantities of ethyl alcohol being produced at the same time. Marquardt, in 1879, advocated aeration of the mash as a means for increasing the production of yeast. With increasing knowledge of the nature of yeasts and their nutritional requirements, better methods for production of baker's yeast have been developed, and a 200 per cent yield on the basis of sugar utilized is now obtained. The concentration and nature of the nutrients in the culture medium are carefully adjusted, growth, for example, taking place in a sugar solution of about 1 per cent rather than 10 to 12 per cent as employed in the fermentation industry. More sugar is added from time to time as needed to keep growth at a maximum rate, over 2,000 lb. of yeast being produced in 12 hr. from an inoculum of 125 lb.

When dough is inoculated with yeast, a portion of the sugar is rapidly

fermented with the production of alcohol and carbon dioxide, this gas causing the dough to swell and giving porosity to the bread produced from it. Yeast will also attack other constituents of the dough, breaking them down into products more readily assimilated by the body or imparting characteristic odors or tastes to the bread. Some of the alcohol and gas is driven off during baking, and most or all of the yeast cells are killed, although bacterial spores will resist the temperature of baking. Spores of *Bacillus mesentericus* are frequently present in flour, and they germinate in bread, particularly in warm weather or when the bread is kept in a warm place. Their development leads to the production of a cantaloupe-like odor and the production of a sticky mass which can be drawn out into threads, hence the name ropy bread. Acids or salts are inimical to growth of *B. mesentericus*, and when trouble with ropy bread is encountered, one or both are frequently added to the bread dough. In most instances the first evidence of the decomposition of bread on standing is the growth of molds, generally *Aspergillus* or *Penicillium* species.

It is apparent that the yeast cell is a complex little "factory" by itself and one capable of performing a considerable number of functions in different environments, its activity supplying some of the essential needs of man as well as some of his pleasures. *Saccharomyces cerevisiae* is the most important single species of the microorganisms of industrial importance, the penicillin producers probably ranking second at the present time.

**Food Yeast.** Chemical analyses of yeast show that it contains about 70 per cent moisture, 13 per cent protein, 10 per cent carbohydrate, 1 to 3 per cent fat, 2.5 per cent mineral matter, and relatively high concentrations of some of the B vitamins, vitamin G, and ergosterol, the precursor of vitamin D. Except for a relatively low fat content, yeast approaches meat rather closely in chemical composition and could be substituted for meat to a great extent if it could be produced cheaply and prepared in an appetizing manner. There are indications that these problems may be solved, and yeast or molds may become of importance in the diet of man. At present, yeast is employed in the diet primarily as a source of vitamins, and in some instances it is incorporated into the diet as a supplement in semifamine areas.

Considerable quantities of brewer's yeast formerly were thrown out as of no value, the bitter components of hops imparting undesirable tastes to it. For this reason it could not be employed in the preparation of bread or as a supplement in cattle food. Methods have been devised for debittering brewer's yeast, and considerable quantities of yeast for use as a food supplement could be obtained from this source. Other yeasts have more agreeable tastes than *S. cerevisiae*. *Tarbia utilis* in particular showing promise as a food yeast. *T. utilis* can use pentose sugars and



organic acids as well as glucose or sucrose, and for this reason it is particularly valuable for yeast production on wood sugars obtainable from the sulfite waste liquids of paper-pulp manufacture. Sulfite waste liquor has a high biochemical oxygen demand and creates a serious problem when dumped into a stream or body of water. Cultivation of *Torula* on suitably treated sulfite wastes markedly reduces the oxygen demand of the liquid, and if the organism can be produced on an economical basis, the process would be an excellent one for both yeast production and waste disposal. Yeast for human food was produced from sulfite liquors in Germany during the Second World War, approximately 16,000 tons being obtained from this source in 1944.

Proteins and fats are the two most expensive major items in the diet of man. Our fat supply is primarily from animal sources and from a few seeds, e.g., cottonseed, corn, and coconuts. Certain species of yeasts and molds can produce considerable quantities of fat under appropriate conditions, and methods have been developed for the production of fat by these organisms. *Endomyces vernalis* produces relatively high amounts of fatty material when cultivated on a medium high in carbohydrate content. *Oospora (Oidium) lactis* is another yeast of potential importance as a fat producer, particularly when cultivated on a whey medium. A number of technical difficulties must be solved before fat can be produced economically by the action of yeasts, but it is becoming apparent that with increasing population on the earth, means must be developed for the utilization of waste materials on an ever-increasing scale. Industrial microbiology can make important contributions in this field.

### MOLD FERMENTATIONS

The term fermentation is employed not only for the anaerobic conversion of organic matter into simpler units with accompanying release of energy but also to partial oxidations of organic compounds under aerobic conditions. The acetic acid bacteria and many of the molds are characterized metabolically by incomplete oxidations; e.g., the acetic acid bacteria oxidize ethyl alcohol to acetic acid. Citric, gallic, and gluconic acids are produced on a commercial scale by the action of different species of molds. This type of fermentation can be illustrated by the citric acid fermentation.

**Citric Acid Fermentation.** Citric acid was formerly obtained primarily from citrus fruits and pineapples, but the demand for it for medicinal purposes, in foods and soft drinks, in candies, and in a number of minor industrial processes has led to the development of large-scale production methods utilizing the metabolic activities of *Aspergillus niger*. Molasses or glucose in 12 to 15 per cent sugar concentration together with amino-



nium, phosphorus, and magnesium salts and trace elements such as iron, manganese, copper, and zinc serves as the culture medium. It is adjusted to a pH near 2.0, sterilized, placed in shallow layers in pans, and inoculated with spores of *A. niger*. During the growth of the mold as a mat on the surface of the medium, a considerable portion of the sugar is converted within the mold cells into citric acid, which is then excreted. Industrial conversions of sugar into citric acid with yields of 60 to 70 per cent can be obtained. Procedures for citric acid production in submerged, vigorously aerated cultures of *A. niger* are being developed. The annual production of citric acid by molds in the United States amounts to over 10 million lb.

Itaconic acid, a raw material of interest for the production of plastics and gluconic acid for pharmaceutical purposes are produced in surface cultures of *Aspergillus terreus* and *Penicillium chrysogenum* (or *A. niger*), respectively, on sugar media. Gallic acid is produced commercially by the action of *Aspergillus* on tannin and is used in the preparation of inks and dyes. Lactic acid (*d* isomer) is also produced to some extent on a commercial basis, species of *Rhizopus* being employed in the process. The utilization of molds for the preparation of certain types of cheese was considered in the preceding chapter.

**Riboflavin.** Butyl alcohol-producing species of the genus *Clostridium* were formerly employed for the commercial production of riboflavin. A number of molds produce this material in higher yields, *Eremothecium ashbyii* cultivated in submerged, aerated cultures appearing to be the most satisfactory one on an industrial scale.

**Enzyme Preparations.** Four principal types of mold enzymes are used on an industrial scale: amylases, invertase, proteinase, and pectinase. Amylases (diastase) act upon starch and are employed in the preparation of adhesives, the desizing of silk textiles, and for pharmaceutical purposes. Invertase, from either molds or yeast, is employed for the production of soft-center chocolates. It hydrolyzes the sucrose in the fondant into glucose and fructose, which imparts a more sirupy consistency to the coated fondant. Proteases are employed for a variety of purposes, degumming silk, tanning leather, preparation of glue, etc. Pectinase is used in the clarification of fruit juices and in the retting of flax, although in the latter case retting is normally carried out by the action of bacteria and other microorganisms rather than by enzymes separated from them.

Koji, actually more a starter than an enzyme preparation, is prepared by the cultivation of *Aspergillus flavus-oryzae* on rice or other grains or on bran. It has marked proteolytic and amylolytic action and is used in the preparation of materials such as soy sauce from soybeans and sake wine from rice.

**Penicillin.** The production of 25.8 trillion units (1,667 units per milligram of penicillin G) in 1946 represented a modern miracle of the application of science and of industrial methods to the production of a material relatively unknown in 1940. The value of penicillin as a chemotherapeutic agent was not recognized before 1940, but once clinical observations indicated the extreme value of this material, a concentrated, cooperative research program was initiated on methods of production. Industrial production of penicillin now ranks as second to ethyl alcohol in value of product produced by microbial action.

Penicillin was first produced by *Penicillium notatum* in a surface-culture fermentation on a carbohydrate medium contained in thin layers in flat bottles. The yield was poor, and an immense amount of labor was required for the production of penicillin. New strains, as well as a related species, *P. chrysogenum*, were found which gave better yields. Improvements were made in the culture medium, it being found that penicillin production was enhanced by the substitution of lactose for glucose, by

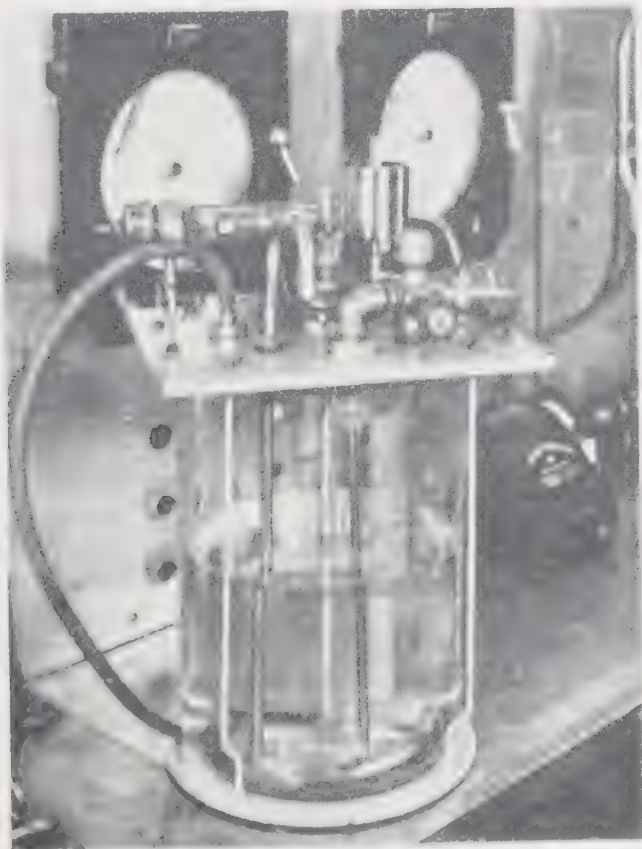


FIG. 12-2. Thirty-liter glass fermenter used for laboratory study of fermentations or production of antibiotics. (Courtesy of Eli Lilly and Co.)

the addition of corn steep liquor (the solution resulting from the steeping of corn during the preparation of starch), by the addition of phenylacetic acid, and by slight changes in the ratio and amount of various ions making up the Czapek-Dox medium commonly employed in the cultivation of molds. The biggest advance was in the development of strains of the mold and methods capable of producing relatively large amounts of penicillin in submerged, well-aerated cultures (see Figs. 19-2 and 19-3) in

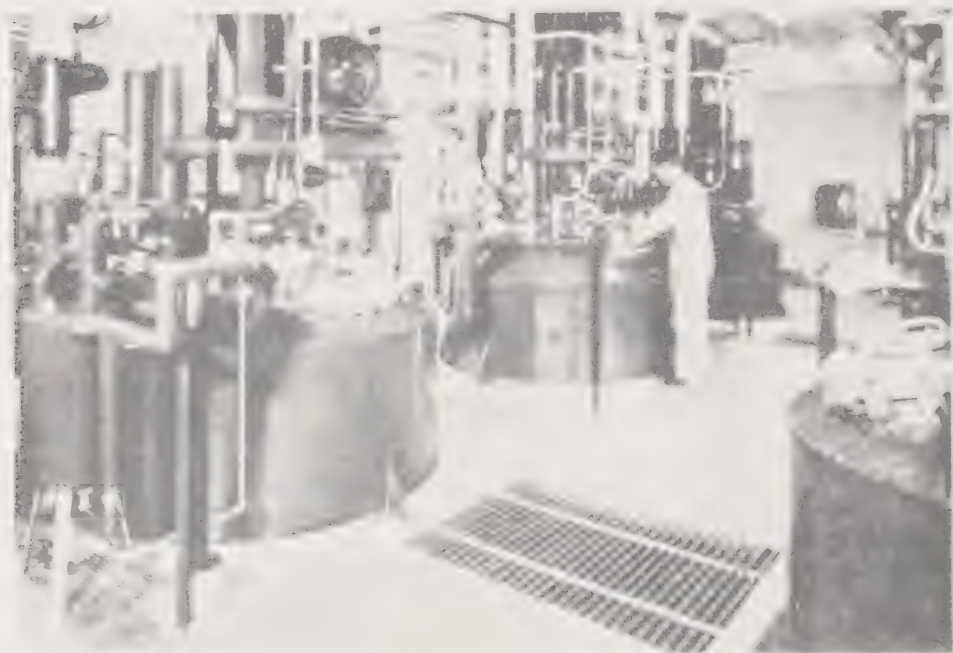
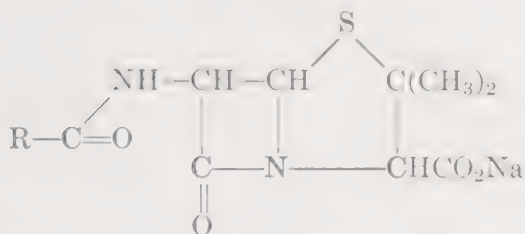


FIG. 19-3 Sixteen-hundred-gallon fermenter for the pilot-scale study of antibiotic production. (Courtesy of Eli Lilly and Co.)

containers of 2,500- to 10,000-gal. capacity, rather than in approximately quart amounts in individual bottles. Time of production was reduced at the same time from 8 to 10 days to 60 to 75 hr. In submerged cultures the mold growth greatly resembles particles of tapioca rather than the type of growth so commonly associated with these fungi. Contamination of the cultures or shifts from suitable environmental conditions markedly inhibit penicillin production.

Penicillin is an extremely unstable substance in acid or alkaline solutions and is most commonly prepared and marketed as the sodium or potassium salt, the purified and dry salts being relatively stable. Actually there are a number of penicillins, all having the same basic formula but differing from each other in the nature of the radical R in the following formula:



In penicillin G, which is commonly employed and which serves as the standard for penicillin activity determinations, the R group has the structure



a benzene ring being attached through a  $-\text{CH}_2-$  group to the structure common to all natural penicillins.

### BACTERIAL FERMENTATIONS

Relatively few bacteria are employed in pure culture on a commercial scale for the production of chemical entities, although many are employed in mixed cultures for the production of cheese and butter, the retting of flax and other textile plants, the tanning of leather, the preparation of coffee beans, cocoa, silage, pickles, sauerkraut, and other materials of economic importance. Bacteria are also used in the preparation of fermented beverages in the warmer countries. Some of the higher bacteria (*Streptomyces*) are employed for the production of various antibiotics. Aerobic culture methods are employed in the production of antibiotics and the aerobic fermentations that lead to the formation of vinegar, dihydroxyacetone, gluconic acid, and sorbose, while anaerobic fermentations are utilized for the production of lactic acid, various solvents, and certain fermented beverages such as pulque.

**Antibiotics.** Streptomycin was the second antibiotic found to have marked value as a chemotherapeutic agent, and is effective against a number of gram-negative and acid-fast bacteria resistant to the action of penicillin. It is produced by *Streptomyces griseus* in submerged, aerated cultures similar to those employed in the production of penicillin. The use of mutant strains isolated following ultraviolet, X-ray, or nitrogen mustard treatment, together with improvements in culture media, have led to higher yields of streptomycin, as is also true for penicillin production. Dihydrostreptomycin is produced by the reduction of streptomycin. Residues from streptomycin production (and from other antibiotic-producing *Streptomyces*) often are relatively rich in vitamin B<sub>12</sub>, and are used as a commercial source of this agent.



Chloramphenicol (Chloromycetin) was produced by *Streptomyces venezuelae* growing in submerged cultures, but this agent can be produced more readily by chemical than by biological methods. The tetracyclines are produced by other species of *Streptomyces*—*S. aureofaciens* forming chlortetracycline (Aureomycin) and *S. rimosus* oxytetracycline (Terramycin), while the parent substance, tetracycline (Achromycin or Tetracycl), is formed by the reductive dechlorination of chlortetracycline. The media employed generally contain sucrose and peanut meal or similar substances, but the details of production of the tetracyclines are, for the most part, trade secrets.

Many other antibiotics are produced commercially, some in processes involving activity of bacteria, but usually these have more limited applicability than penicillin and the antibiotics mentioned above. A few of these miscellaneous agents may prove to be of value in the treatment of diseases of viral, rickettsial, or fungal origin, but most are employed for topical application to man, control of plant and animal diseases, and food supplements for animals. Moore reported in 1946 that succinyl-sulfathiazole or streptomycin added to a purified diet for chicks led to increased growth of the chicks. Others observed the same response in chicks, turkeys, and various other animals. Large amounts of antibiotics are employed as adjuncts in animal feeds but for the most part at a level on which they have little antibacterial activity. Their mode of action in stimulating growth of poultry and animals is not understood. The antibiotic industry has grown by leaps and bounds from practically zero production in 1940 to over 2,000 tons in 1955, with a value of around \$250,000,000. Search for new antibiotics, and for improvements in methods of preparing the known ones, continues.

**Vinegar.** When fruit juices are allowed to stand, they generally undergo alcoholic fermentation, a concentration of alcohol being produced which is inhibitory to further growth of yeasts and also to the growth of many species of bacteria. Species of the genus *Acetobacter*, however, are capable of growing on the surface of wine or cider, obtaining a considerable portion of the energy required for growth from the oxidation of ethyl alcohol to acetic acid according to the equation



When wine or cider is allowed to stand in an open container, the acetic acid bacteria will in time contaminate the material and grow, in association with yeasts, on the surface with the formation of a thick, jelly-like layer known as mother of vinegar. Pasteur recognized that beer and wine frequently spoil on standing owing to acidification by microorganisms. Hansen, a Danish bacteriologist, isolated and described two species

of the acetic acid bacteria, naming them *Bacterium* (now *Acetobacter*) *aceti* and *B. pasteurianum*. Other *Acetobacter* species are present in vinegar, pure cultures of the desired species or strains now being employed on a commercial scale to obtain the desired odor and flavor produced by the action of the organism on constituents of the wine or cider acetified.

When the vinegar is exposed to air for a time, the acetic acid bacteria may slowly oxidize the acetic acid to carbon dioxide and water, certain species being more active than others in oxidizing to completion. These organisms are not active under anaerobic conditions, and the 4 to 5 per cent acetic acid content of vinegar is quite inhibitory to the growth of other bacterial species. Vinegar is generally pasteurized to prevent continued activity of the acetic acid bacteria and the growth of other organisms.

The acetic acid bacteria are characterized by the incomplete oxidation of alcohols and of sugars and by their extreme pleomorphism, filamentous and large club-shaped forms frequently being encountered. Many but not all species form a thick zoogloal membrane when cultivated on liquid media. They are widely distributed in nature, where they are generally associated with the souring of fruits.

Commercial production of vinegar is carried out in large, partially filled casks, in which the bacteria grow in a film (often supported by wooden floats) on the surface of the wine or cider, or in generators in which the medium trickles over wood shavings. A heavy film of acetic acid bacteria develops on the shavings, and aerobic conditions are maintained by a flow of air in the direction opposite to that of the liquid being acetified. This method permits continuous operation, but it is claimed that the product is not of so high a quality for table use as that produced by the slower fermentation in casks.

The acetic acid bacteria are also utilized to a limited extent for the oxidation of the sugar alcohol, sorbitol, to sorbose, which is employed in the production of vitamin C, and in the oxidation of glycerol to dihydroxyacetone. *Acetobacter suboxydans* is generally employed for these oxidations, this species showing little tendency to carry the oxidations further.

**Lactic Acid.** We have considered in the previous chapter the production of lactic acid during the souring of milk. On the industrial scale, lactic acid itself is prepared by the fermentation of lactose in whey, of sucrose in molasses, or of glucose in starch hydrolyzates. Any of several species of the lactic acid bacteria can be employed, although *Lactobacillus delbrueckii* is generally the organism of choice. The fermentation is carried out around 50°C., as this organism is somewhat thermophilic and the relatively high temperature is inhibitory to the growth of most other

species of bacteria. The use of this temperature also reduces the cost of production since the medium need be only pasteurized rather than completely sterilized, unless marked contamination with anaerobic spore-formers is encountered.

Calcium carbonate is added during the course of the fermentation to neutralize the acid as it is formed and thereby encourage further growth of the bacteria. Lactic acid yields equivalent to 85 per cent of the sugar fermented can be obtained on a commercial basis. Calcium lactate can be crystallized from the filtered and concentrated fermentation liquor and converted to lactic acid on acidification with sulfuric acid, the calcium being precipitated as the sulfate. This acid can be further purified by appropriate chemical methods. Annual production of lactic acid is in the neighborhood of 10 million lb. A recent development indicates the possibility of producing lactic acid from sulfite waste liquor, a process which, if successful on a commercial scale, would be of value in the disposal of sulfite wastes, which at the same time would serve as cheap starting material. Lactic acid is employed in the tanning industry, in the food industry, in a number of pharmaceutical preparations, and in the preparation of plastics.

**Butyl Alcohol-Acetone Fermentation.** Pasteur was the first investigator to show that butyl alcohol is a product of microbial fermentation along with butyric acid and other substances. Acetone was later found to be produced during the course of the butyric acid fermentation. Acetone was needed in considerable amounts during the First World War for the production of explosives and airplane "dopes," and methods for the industrial production of this material by fermentation were developed. The demand for acetone diminished after the war, and chemical methods were developed for its production, both factors resulting in diminished interest in the microbiological aspects of its production. Since then, an increasing demand for butyl alcohol (butanol) in the production of lacquers has developed, and research has been directed primarily to the production of this substance.

Butyl alcohol can be produced from a variety of sugar or starch sources and by a number of bacteria, the two important industrial species being *Clostridium butylicum* and *C. acetobutylicum*, the latter species being particularly valuable in starchy media. The fermentation is a complex one; end products varying with time in a manner illustrated in Fig. 10-6. The butanol fermentation is biologically unstable, and much attention has been given to the selection and maintenance of high-producing strains. It has been observed that the vegetative cells derived from the more heat-resistant spores are frequently the most active fermenters. "Heat-shocking" has therefore been used in the preparation of the inocula for start-up, the culture being heated for 1 to 2 min. at 100° C. to kill the cells and

the less resistant spores. The fermentation is also markedly influenced by contaminants which influence the course of the fermentation. Ethyl alcohol and lactic acid fermentations have a considerable factor of safety as regards the production of the desired products, a factor not present in the butanol and penicillin fermentations. In the latter industries, extreme care must be exerted to exclude other organisms, and the technique of preventing contamination on a large scale is an extremely difficult one. In addition to being influenced by other bacteria, the butyl alcohol bacteria are also subject to the action of bacteriophages specific against them. The producer must use extreme care in all manipulations, and it is necessary to be thoroughly familiar with the nutritional requirements of the strain employed, a rapid and vigorous fermentation giving highest yields with least trouble.

A mixture of end products is produced, a typical fermentation of 1,000 parts of starch yielding 220 lb. of butanol, 85 of acetone, 15 of mixed alcohols, 520 of carbon dioxide, and 14 of hydrogen. The hydrogen and carbon dioxide are collected and can be catalytically converted into methyl alcohol. Considerable amounts of riboflavin are formed, and this fermentation was formerly a main source of this vitamin. Acetyl-methyl-carbinol is another minor product of the fermentation.

In concluding this summary of the industrial activities of bacteria it might be well to consider that agriculture is one of the most important industries and that the microbiological activities in the soil constitute the most essential industrial application, fortunately one that does not require the extreme care needed in the factory. For other industries, man selects an organism contributing its mite in the economy of nature, an organism producing an agent of economic value to him, and harnesses it as well as he can to do his purpose, at times in conflict with other organisms which find the environment suitable for their own ends.

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## CHAPTER 20

### INFECTION AND RESISTANCE

In preceding chapters we have been considering the activities of bacteria in various inanimate environments. At this time we shall turn to a consideration of the more complex relationships observed when bacteria are parasitic on man or other animals. The discussion will be limited primarily to the relationships between pathogenic species and their hosts which lead to the development of an infectious disease or of recovery therefrom.

**Disease.** The healthy organism is one so adjusted that it is normally capable of carrying on all the functions necessary to its existence. These functions are guided by the metabolic and other processes of the various cells which constitute the plant or animal. Once the rather indefinite limits of the healthy state have been passed, the individual is said to be diseased. Hence disease is an abnormal state or condition of the individual organism, or parts thereof, and wide variations may be observed in the extent of the diseased state. An abnormal state of a plant or animal may be induced by a variety of factors, physical, chemical, or biological in origin. These may be classified and illustrated in a general way as follows:

1. Physical agents: heat, cold, pressure, or radiations
2. Chemical agents: chemicals present in marked excess or possessing specific toxicity  
Lack of essential substances such as vitamins
3. Biological agents: saprophytes or parasites which have entered another organism and produced an abnormal state, an infectious disease

In the case of infectious disease, the invading parasite in some manner interferes with the normal sequence, rate, or type of metabolism and other processes of a group, or groups, of cells. Tissues of the host provide substrates for the parasite to feed upon. The ability of the parasite to utilize these materials under the conditions prevailing in the host is an important prerequisite for growth and proliferation of the invader, i.e., establishment and maintenance of the disease state. Final understanding of the true nature of the various infectious diseases depends on a full understanding of the normal functions and activities of the para-

sitic cells, of the cells of the host, of the interrelationships between the cells of the parasite and of the host, and finally of the abnormality of function elicited as a result of this host-parasite association. Needless to say, most of these are unknown factors, but we shall attempt to summarize the general knowledge of the interplay between the pathogenic parasite and its host.

**Early Concepts of Disease.** A number of hypotheses were advanced during early times to explain the nature and cause of disease. In one hypothesis, the demonic, it was postulated that evil spirits invaded the body and produced damage therein. Charms, spells, and incantations were invoked to keep the evil spirits away or to drive them out once they had invaded the body. Other peoples postulated that disease was the result of acts which brought forth the displeasure of the gods and was meted out to the individual as punishment for his sins. A more recent suggestion was that disease originated in dirt or filth and could be transmitted to a healthy person. An earlier idea along somewhat similar lines held that disease is caused by miasmas or vapors originating from swamps or bogs. These latter concepts were based on observations that disease was more prevalent in lowlands. Observations of this nature led to philosophical considerations in which it was suggested that microorganisms might be present in such material and that they were the cause of disease. Actual evidence for this hypothesis began to accumulate during the early part of the nineteenth century.

During this time the search for the presence of microorganisms in diseased tissue was prosecuted with enthusiasm. A number of workers attempted to produce infections in experimental animals by the transfer of such matter, and in many experiments they were successful. However, much of this early work was of a sloppy character, could not be readily confirmed, and as a result the "germ" theory of disease was in general disrepute. We must remember that experimental methods in microbiology were very crude at this time, and the lack of pure cultures was a serious handicap. Jacob Henle, a pathologist, in 1840 protested against the poor experiments and loose reasoning of the time and demanded that evidence be submitted which would show that the microorganism suspected of being the infectious agent of the contagion (1) could be constantly found in the infected tissue, (2) could be isolated from it, and (3) on injection could induce the disease. Almost thirty years elapsed before these conditions were met and it was definitely established that microorganisms could produce infections in animals and plants.

A number of workers around 1850 observed the presence of relatively long bacteria in great numbers in the blood of animals dead of anthrax. This led them to suspect that these rods might be the etiological agent of the disease. The studies of Davaine in the period 1863 to 1868 gave con-

siderable support to this belief, particularly when he was able to demonstrate that anthrax can result from the inoculation of a healthy animal with as little as one-millionth of a drop of blood from an infected animal. He was strengthened in this belief by the studies of Pasteur on the microbe specificity of fermentations and of microorganisms as the cause of diseases of wine and beer, reasoning by analogy that diseases of plants and animals might also be caused by specific microbes.

In 1865 Pasteur was commissioned by the French government to investigate a disease, pébrine, of silkworms, which was seriously crippling the French silk industry. He presented considerable evidence that the disease was caused by a protozoan organism and that a second disease, flacherie, was induced by bacteria. He was also able to introduce methods for the control of these infections, and as a result of these studies he became interested in the study of infectious diseases and in particular in methods for their control.

In 1876 the German doctor, Robert Koch, was able to demonstrate that the bacterium observed in such large numbers in animals dying of anthrax was actually the etiological agent of this disease. He succeeded in isolating the organism in pure culture in sterile vitreous humor from the eyeballs of cattle, passing it a number of times in the laboratory, and reproducing the infection in experimental animals inoculated with pure cultures of the anthrax bacillus. This study (confirmed by Pasteur in 1877), together with one on the microbial causes of wound infections reported in 1878, Koch's discovery in 1882 of the tubercle bacillus, and the demonstration that it is the causative agent of tuberculosis, firmly established the theory that a particular infection is induced by a specific organism. In the study of tuberculosis Koch set forth his famous *postulates*, which should be fulfilled if an organism is to be definitely considered as the causative agent of a disease. These, in essence an amplification of the reasoning of Henle, one of Koch's teachers, may be stated as follows:

1. The organism must be present in all cases of the disease
2. The organism must be isolated and cultivated in pure culture
3. The specific disease must be induced in susceptible animals following the injection of the pure culture
4. The organism must be present in typical distribution in, and be recoverable from, the experimental host

During this time and in the next few years Koch and other workers introduced a variety of technical methods for the study of microorganisms, and these, together with the impetus provided by the earlier observations of Koch and of Pasteur, were responsible for rapid progress in the isolation and identification of various microorganisms as the causative agents of a wide variety of infectious diseases of both plants and animals.

At the same time it became apparent that certain diseases are caused by factors other than microbial in origin. It is with the causative agents of infectious disease, the pathogenic microbes, and their interplay with their hosts that we shall be concerned.

### BIOLOGICAL ASPECTS OF INFECTIOUS DISEASE

We are apt to think of infectious diseases as states peculiar to ourselves, and incidentally to other forms of life, and to forget the broader biological principles involved. Predatory forms of life prey upon other animals for nourishment, and this is considered a normal event in nature. Likewise the consumption of plants is regarded by the consumer as a beneficial process, but would this concept be shared by the plant or animal consumed? The main object of life of a nonthinking organism appears to be maintenance of self, i.e., avoiding enemies if possible, securing sufficient food, and reproducing the species.

A parasite is an organism which resides on or within another living organism, the most successful parasites achieving a balance with the host in which survival, growth, and reproduction of both does occur. We have seen that both participants in such a symbiotic, parasitic relationship can be of marked aid to each other, e.g., termites and their intestinal protozoa which digest cellulose. Neither can live without the other. Less dependent relationships are noted between man and his intestinal flora—*Escherichia coli*, for example, contributing little to its host and even under some conditions exerting a pathogenic effect in man, generally as a secondary invader or opportunist. Changes in the intestinal flora induced by chemotherapeutical or other agents may, however, result in marked discomfort to the host. Other parasites of man may be truer commensals, particularly those living on the skin or on membranes of the respiratory tract and contributing little or nothing to their host. Such parasites may be facultative ones or they may be obligatory parasites.

The aim of parasitic life is similar to that of predatory life, an attempt to secure a supply of food under environmental conditions suitable for the maintenance of the species. It has been postulated that the parasites originated from saprophytic ancestors which in the course of time became adapted to growth on the dead tissues of another organism. In the course of evolution by adaptation, the parasite finally became adjusted to growth on or in living tissues and lost many of the powers of synthesis possessed by its ancestors. This loss of synthetic powers appears to reach its extreme in the case of the filtrable viruses, it having been suggested that these agents "borrow" certain characteristics of life from the cells which they parasitize. Hence the parasite becomes more or less dependent upon its host, frequently a specific one, and this dependence



in some instances is a skillful one, while in others the parasite is a bungler and produces such an amount of destruction frequently in a short time that it destroys its host. Not only must there be a specific host, or host range, but there must be, for the parasite, suitable portals of entry and of exit from the host and also an effective mechanism for the transmission of the parasite to uninfected hosts, if the parasite is to survive.

Bacteria, or other microorganisms, may be parasitic on the body surfaces, external or internal, and do no damage; either they are unable to invade the deeper tissues, or if they do gain entrance, they are quickly destroyed. Some of these external parasites do at times establish themselves within the host, elicit damage, and are then considered pathogenic parasites. Other pathogenic species may never be found on or in the normal body, their existence being dependent upon transfer from one susceptible individual to another. Still others may be pathogenic in one species while they grow as harmless parasites upon another host type and are unable to maintain themselves at all in still other species. Thus there is considerable *host specificity* manifested by many parasites, and this tends to be the rule amongst the pathogenic forms, suggesting a highly specialized adaptation to the host or host range.

The simplest animal, an amoeba, has learned that other cells may serve as a source of food. Amoeba frequently flow around bacteria and then proceed to digest the ingested organism in a vacuole into which the amoeba secretes digestive enzymes. When the amoeba succeeds in converting the ingested bacterium into assimilable foodstuff, we consider it as a simple act of digestion. At times the ingested bacterium may resist digestion by the amoeba, and the latter generally flows away from its unwilling banquet. But let us suppose that the bacterium started to digest the amoeba rather than the reverse situation which was considered above. So far as the amoeba is concerned, this would represent an unhealthy state, an infectious disease, while from the viewpoint of the bacterium it is simply an act of digestion. We see in this simple illustration that both offensive and defensive factors are involved in the interrelationship between unicellular forms, factors which become more complicated when a higher form of life is involved. Just as the animal preyed upon has certain defensive forces—cunning, camouflage, and protective coverings—against the predator, so likewise the host has nonspecific, and may develop specific, protective mechanisms or agents against the invading parasite. The general nature of the offensive forces of the pathogenic parasites and of the defensive forces of the host will be summarized in the following sections.

**Offensive Forces of the Parasite.** It was implied above that *infection* may be regarded as the establishment of one organism within the tissues of another with subsequent damage to the latter. It should be pointed

but that contamination of one organism with another does not constitute infection; the contaminant must become established if a true infection is to develop within the host. An infection may be a localized one, or the invading microorganism may spread through the lymphatics and the blood vessels to other tissues or organs of the infected host. Any organism which can produce an infection or infectious disease is said to be pathogenic or to be a pathogen. These terms imply only that the organism is capable of producing an infectious disease in another, without any reference to its facility in doing so or to the severity of the resultant infection. The pathogen may or may not be able readily to induce an infection, and the infection produced can be mild or severe in character. Another term, *virulence*, is therefore commonly employed to denote a more quantitative measure of the ability of a pathogen to produce an infection in a definite host. Thus we speak of a pathogen as an organism of low, intermediate, or high virulence according to its ability to by-pass or overcome the defensive mechanisms of a specific host. Virulence is expressed at times in terms ( $LD_{50}$ ) of the minimum number of organisms which, following introduction by a particular route into a specific host, will result in infection and death of 50 per cent of the test animals. Both the virulence of the pathogenic parasite and the resistance of its host are subject to variation and the occurrence of infection, or of recovery therefrom, is determined by the balance established between the offensive forces of the invader and the defensive mechanisms of the invaded.

The virulence of bacteria depends upon two general factors: (1) their invasiveness or aggressiveness, the ability to invade and multiply within the tissues of the host with subsequent damage to the host, and (2) damage done to the host by poisonous agents, toxins, excreted by or present in the parasite. In the case of anthrax, for example, virulence is almost completely associated with invasiveness and heavy growth of the anthrax bacillus; in the case of tetanus, with toxin produced by the tetanus bacillus, which has little or no invasive power, and in still other organisms there are numerous examples of intermingling of the two factors of virulence.

**Invasiveness.** The invasive power of microorganisms involves their ability to penetrate surface barriers—the intact skin and mucous membranes of the host—and to multiply within and spread through the tissues once invasion has been accomplished. Certain pathogenic forms are able to live on these barriers indefinitely; others may be killed within a short period of time by materials present in the normal secretions of the skin or mucous membranes. Staphylococci are normal parasites on the skin of man, while the typhoid-dysentery group of bacteria can generally be removed only during the first few minutes after contamination of the skin has occurred. Many pathogenic species are never able by them-

selves to penetrate the epithelial barriers, and they enter the deeper tissues only when the mechanical or chemical barriers to their entry have been broken by injury, either mechanical or biological in origin. Also, there generally are definite vulnerable portions of the host, portals of entry, through which specific pathogenic microbes gain entrance to the deeper tissues or organs. As illustrations we might consider that the pneumococcus ordinarily invades the respiratory tract, the gonococcus the genital tract, and the typhoid-dysentery group the intestinal tract, and that these organisms may not be able to establish themselves if introduced by other routes.

After bacteria have gained entrance to the tissues, they attempt to multiply therein but are opposed by defensive mechanisms of their unwilling host. When the invader is a saprophytic form, it is quickly removed and destroyed by amoeba-like cells known as *phagocytes*. The fact that pathogenic forms are, in many instances, not readily taken up by the phagocytes suggests that the former possess a defensive mechanism against the phagocytes; in other words, this particular offensive force is a part of their characteristic virulence. The capsules possessed by pathogenic species such as the pneumococci in their smooth, virulent form apparently inhibit phagocytosis. Other pathogens may form chemical agents, leucocidins, which destroy the phagocytes, while still others liberate substances which repel the phagocytic cells, an example of negative chemotaxis.

Once the bacteria have passed the mechanical barriers to their entry, they may also encounter blood or tissue fluids which possess bacteriostatic or bactericidal activity against various pathogens. When the pathogen has the ability to neutralize the inhibitory action of the body fluids, it would be considered as another offensive mechanism of the parasite. In addition, many bacteria have the ability to destroy red blood cells by means of a hemolysin secreted by the pathogen, and such an agent, if active in the body, would tend to decrease the general state of health of the host and render him more susceptible to invasion by the parasite. Also the fibrin clot, which is deposited by blood at the site of an injury and which serves to wall off the injured portion from the rest of the body, may be attacked and dissolved by fibrinolysis (streptokinase) produced by a variety of bacterial species, particularly streptococci.

Other agents elaborated by a number of pathogenic bacteria include the spreading factor of Duran-Reynolds and the necrotizing, or cell-destroying, toxins. The former has been identified as an enzyme, hyaluronidase, which has lytic action upon carbohydrate material (hyaluronic acid) found between the cells of a tissue. By its lytic ac-



tion, hyaluronidase may aid in preparing an intercellular pathway of invasion. The necrotizing toxins in some manner bring about the death and necrosis of the tissue cells, thereby providing dead organic matter which can serve as foodstuff more readily utilizable by the pathogen.

In the above discussion only the more important agents associated with the virulence of pathogenic bacteria have been considered. There is no doubt that other agents or properties will be recognized in the future and that knowledge of the known factors will be enlarged and clarified. At the present time, it is known that these agents or properties do exist, but it is impossible as yet to assess their full significance in the struggle between the invading parasite and the invaded host. It may well be that the enzymic composition of the parasite plays an important part in its aggressiveness, the nature and activity of the enzymes controlling not only microbial nutrition but also the formation of chemical agents which may be inimical to the defensive mechanisms of the host.

**Toxicity.** Virulence of a parasite may also be associated with its ability to produce specific toxins, an ability which likewise is associated with the enzymic structure of the pathogenic organism. In general, the toxins are excreted by the cells and are found free in the medium in which the cells have multiplied; hence these poisons are spoken of as *exotoxins*. We have already mentioned the necrotizing exotoxins, which may aid the spread of the organism through the tissues. Other exotoxins are generally not associated with invasiveness and exert their poisonous effects on tissues or organs remote from the site of the infection. The exotoxins of bacteria belong to a group of chemical agents having very characteristic properties, all being rather unstable, complex, proteinaceous compounds of high toxicity which is generally exhibited only after a characteristic incubation period in the body. This is in sharp contrast to the rapid action of the common poisons. After repeated inoculations of sublethal doses of a toxin into an experimental animal, the latter develops a specific neutralizing agent known as an antitoxin and becomes highly resistant to massive doses of the toxin.

Diphtheria is an infectious disease in which the characteristic symptoms are primarily due to the exotoxin produced by the bacterium *Corynebacterium diphtheriae* multiplies in the superficial tissues of the tonsils and may spread by continuity over adjacent tissues. Ordinarily the local infection is never extensive, and the severe nature of diphtheria is associated with the action of diphtheria exotoxin, which is formed at the site of the infection and absorbed by the blood. It might be argued that toxin formation should not be considered a part of the virulence mechanism of the diphtheria bacilli, since the liberation of toxin ordinarily results in the death of the infected individual. Possibly it should be



regarded as bungling on the part of the parasite, but toxin formation does contribute to the diseased state and is therefore commonly included in a discussion of virulence.

As a rule, those bacteria characterized by exotoxin production show very little invasive power and usually multiply only to a limited extent within the body. Those pathogenic bacteria which do not form exotoxins ordinarily give rise to more extensive invasions of the host and multiply to a much greater extent within the tissues when the infection is a severe one. The actual presence of large numbers of bacteria within the body tissues or fluids appears to be a prime factor in the production of injury by certain pathogens, and in fact in some instances the bacteria need not be alive. Intoxication or death may follow the injection of suspensions of dead bacteria. This was commonly explained on the assumption that organisms undergo disintegration within the body with liberation of toxic materials known as *endotoxins*. These rather hypothetical substances are not as toxic as the exotoxins, in general are not capable of eliciting antitoxin production, and are probably not preformed agents within the bacteria but rather cellular degradation products produced therefrom. In the case of the typhoid bacillus and many other gram-negative bacteria, the toxic agent appears to be a carbohydrate-lipoid complex in association with bacillary protein.

**Variations in Virulence.** The virulence of pathogenic bacteria is subject to variation. When a particular species is passed a number of times in laboratory media, its virulence tends to decrease and may ultimately disappear, the original parasite becoming primarily saprophytic in character. Only those bacteria best suited for growth on laboratory media tend to survive. On the other hand, transfer from a culture to a susceptible animal tends to aid the organism in maintaining its virulence. Frequent transfer from one animal to another of the same species may enhance the virulence of the organism for that animal species. Virulence, as far as species specificity is concerned, may also be altered on passage from one animal species to another. A bacterium pathogenic for rabbits may not be markedly virulent for guinea pigs on first inoculation into the pig, but on repeated transfer from pig to pig, virulence may be enhanced for the guinea pig and be decreased for the rabbit. This phenomenon of enhanced specificity for a particular host is more pronounced with the filtrable viruses than with the bacteria. Virulence is not a property of the pathogenic parasite alone but is also associated to a considerable extent with the nature of the host. Virulence must not be considered as a permanent intrinsic property of the species; it is subject to variation and is actually only a relative expression of the ability of a particular strain of the infectious agent to produce an unhealthy state in a specific host under a definite set of conditions. Variations in virulence markedly in-

crease the difficulties encountered in the study of infectious diseases and, together with variations in the resistance of a host species, introduce variables which are difficult to control in experimental studies. In the broadest biological sense these variations are simply examples of the ability of an organism to adapt itself to changes in its environment, whether the adaptation occurs by changes in enzymic pattern, by alterations in cell structure, composition, or function, by the selection of strains best suited for growth under a given set of conditions, or by unknown mechanisms. Selection of mutants best adapted to the environment appears to be the major factor.

**Defensive Forces of the Host.** We have considered that an infectious disease is the result of a struggle between a parasite and its host. This struggle implies that the parasite has not successfully adapted itself to the host species and that the host has certain defensive mechanisms against the parasite. Many potential hosts for lower organisms do not provide a suitable pabulum or temperature for growth of the latter, and adaptation never occurs. Such species are said to be *naturally immune* to particular species parasitic upon other forms of life. When natural immunity is a characteristic of a given species, it is frequently spoken of as *species immunity*. There is evidence that differences in immunity exist between different races of the same species, giving rise to *racial immunity*. There is also evidence that immunity may vary in members of the same race and even in the same individual with changes in age, nutrition, mode of living, or general state of health. This suggests that immunity is not an absolute state and that it must be considered as ranging from complete resistance against a lower organism to a degree of resistance only slightly greater than that normally possessed by the species. We cannot consider these various ramifications of immunity or resistance in a text on general bacteriology and shall devote our consideration to the general lines of resistance or defense of a host against a species pathogenic for it.

**The First Line of Defense.** In previous remarks, various defensive mechanisms of the host were mentioned in connection with the discussion of the offensive forces of the parasite. At this time, let us consider the defensive forces of the host from the viewpoint of the host rather than of the parasite. Most bacteria are unable to establish themselves on the skin and mucous membranes, which constitute the first line of defense of the body against invasion by foreign matter. The environment is not suitable for them. Furthermore, the intact skin and mucous membranes serve as mechanical barriers and may be aided at times by natural secretions exerting bacteriostatic or bactericidal activity. Reflexes of various sorts may also serve as a defensive mechanism, sneezing expelling foreign matter from the nostrils, vomiting expelling irritating

matter from the stomach, and diarrhea serving a similar function in the removal of toxic matter from the intestinal tract. A few microbes have developed the ability to evade the protective devices, but for the most part the latter are successful in the prevention of infection by the countless contaminants to which the body is exposed during the life of the individual.

**The Second Line of Defense.** Once a contaminant has successfully passed the first line of defense, either by means of its own offensive forces

or by taking advantage of breaks in the external defenses, it is subject to attack by fixed or wandering phagocytic cells of the body. These cells, possessing the ability to engulf and to digest foreign matter, constitute the second line of defense. The protective mechanism, *phagocytosis*, which they exert was first observed by Metchnikoff, a colleague of Pasteur, in 1884. Metchnikoff observed that *Daphnia*, a transparent "water flea" which was present in his aquariums, frequently ingested small, thin, pointed spores of a fungus also present in the aquariums. The spores could be observed in the digestive tract of this crustacean, and Metchnikoff noted that occasionally the spores penetrated the walls of the



FIG. 20-1. Elie Metchnikoff, founder of the cellular theory of immunity.

digestive system, entering the deeper tissues of the water flea. Here they were engulfed by certain wandering cells which he called phagocytes (cells which devour) and which ordinarily destroyed the fungus spores. But on occasion these spores did germinate within the phagocyte, destroy the latter, and in time bring about death of the flea--again an act of digestion, or an infectious disease, depending on whether the point of view is that of the fungus or of the crustacean.

Metchnikoff had the brilliant idea that similar cells, either free or fixed, in the animal body could ingest foreign matter including invading microorganisms and remove or destroy the invader. These observations led to the development of the *cellular theory of immunity*, in which it was postulated that resistance to an invading organism depends primarily on phagocytic activity, the animal being considered immune if its phagocytes were capable of ingesting and destroying the invading organism. Failure of the phagocytic system would lead to development of the invader and the establishment of an infectious disease.



Most of the infections which remain localized illustrate the action of this second line of defense. *Micrococcus pyogenes* var. *aureus* lives as a harmless parasite on the skin of man but may gain entrance to the body through a scratch or other injury, and thereby the deeper tissues become contaminated. When these staphylococci multiply and establish themselves within the injured tissues and in the blood clot, an infection is established. The edges of the affected area exhibit slight redness, localized swelling is observed which becomes more pronounced with time, and the area becomes painful to touch. The site of injury also fills with pus, a yellowish exudate containing numerous phagocytic cells. Normally the redness, swelling, tenderness, and pus disappear with time, as the staphylococci and cellular debris are removed by phagocytic action and normal tissue repair occurs. Occasionally the staphylococci will penetrate deeper into the tissues and may spread through the body, producing a generalized infection. What happens during the course of the infection?

As the staphylococci multiply, they obtain nutrients from the tissues of their unwilling host, generally from damaged cells, from the blood clot, and from tissue excretions at the site of the wound. Conditions for multiplication are favorable, and the invading parasite takes advantage of its opportunities, employing the offensive factors, such as the liberation of substances which injure or kill adjacent tissue cells, which it possesses.

As a result of the injury and infection, the tissue cells recognize the presence of material foreign to themselves, "not self" in the terminology of Burnet, and attempt to counteract the presence of the foreign material. The tissues liberate chemical substances (such as histamine) which cause the blood capillaries supplying the area of the wound to relax, thus inducing greater blood flow to the area with consequent reddening. Relaxation of the capillaries renders them more porous, and additional fluids seep from them into the affected area producing a swelling. At the same time the phagocytic cells (also called leucocytes, or white blood cells) pass through the walls of the capillaries and migrate to the site of the infection, probably being attracted to the site by chemical agents liberated by the injured tissues. Such an attraction is spoken of as positive chemotaxis and is the opposite of the behavior exhibited by virulent bacteria possessing the ability to repulse phagocytic cells. The general type of response, as considered above, of the host to the presence of foreign matter is known as *inflammation*, or the *inflammatory process*, and can be considered as a defensive mechanism, part of the second line of defense.

The dead tissue cells tend to assume properties of not self and are soon engulfed by the phagocytes, which at the same time tend to wall off the living tissue from the injured portion. Behind this barrier, normal tissue



cells attempt to repair the damage caused by the mechanical injury and that produced by bacterial action. The phagocytes also attempt to engulf and digest the invading bacteria, but in this biological warfare the latter resist phagocytic action to the best of their ability. Microscopic observation of the pus at this time would show that it consists primarily of bacterial cells and phagocytes, many of which may have been destroyed in the struggle. Highly virulent staphylococci may succeed in penetrating the phagocytic barrier and invade deeper tissues, particularly the lymph glands. The struggle for existence on the part of the parasite and of the host continues, with other types of phagocytic cells, generally ones fixed in the tissues rather than being free in the blood stream, entering the combat. The outcome of the struggle depends to a great extent upon the relative values of the offensive forces of the parasite and the second line of defense of the host, the phagocytic system.

**The Third Line of Defense.** In pneumococcal pneumonia we might observe a sequence of events similar to that outlined for a staphylococcal infection. As the infection continues in the lungs, the struggle reaches a crisis followed by death or by recovery of the stricken individual. What was responsible for recovery from an untreated pneumococcal infection?

Early in the course of the disease, microscopic observations would indicate that the pneumococci were relatively resistant to phagocytosis. Virulent strains of pneumococci are encapsulated, and we have seen that the possession of a capsule by these forms appears to inhibit phagocytosis. The capsular material may be somewhat similar to tissue material and as a result interferes with "self-not self" determination on the part of the phagocytes, a conjecture, to be sure, but possibly one of the many factors involved in the interrelations between parasite and host. About the time that improvement is noted in the stricken individual, it can be demonstrated that the phagocytes readily ingest the pneumococci. Why? Have the pneumococci, the phagocytes, or both undergone change, or are still other factors involved?

Suppose that there is available a quantity of phagocytes, of the pneumococci responsible for the infection, and three samples of serum (the clear liquid obtained after the blood has clotted) from the diseased person, one sample of serum obtained before the infection, one taken early in the course of the disease, and the third after recovery was under way. It could be demonstrated (see Fig. 20-2) that the phagocytes do not readily engulf the pneumococci in the presence of serum taken before or early in the course of the infection. Phagocytosis does occur when material from the same samples of phagocytes and of pneumococci are mixed with serum obtained at or after the crisis in the course of the disease. This would suggest that an agent which stimulates phagocytosis

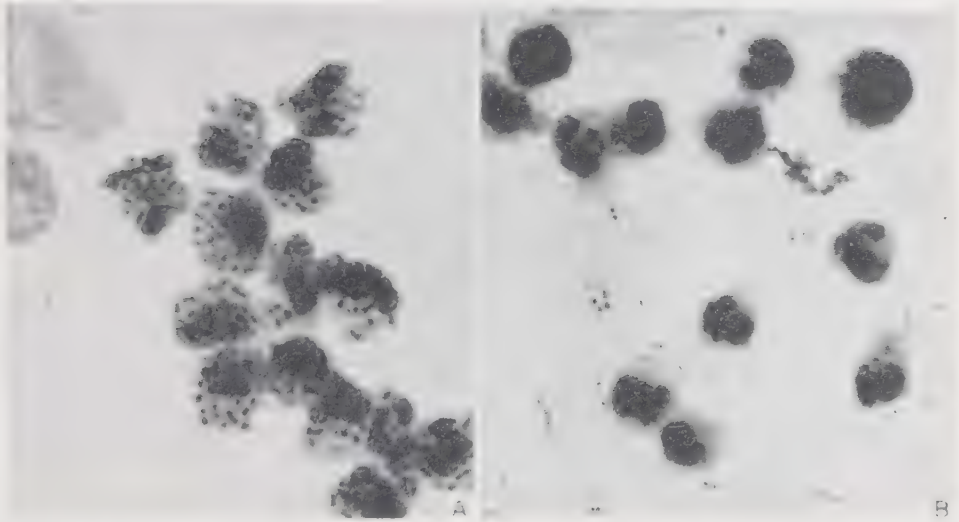


FIG. 20-2. Influence of immune serum (antibodies) on phagocytosis: (A) phagocytes + immune serum + pneumococci; (B) same as (A) except that normal serum was substituted for immune serum, and phagocytosis is not noted. (Courtesy of S. Raffel.)

is present in the blood of the individual recovering from pneumonia. This conclusion would be further supported by the demonstration that there had been no detectable change in the activity of the pneumococci or of the phagocytes of the individual during the course of the infection, when the phagocytes were tested in the presence of normal serum. The only variable in the experiments, therefore, is in the nature or composition of the individual's blood. Other evidence indicates that a chemical agent (or agents) is formed by the host during the course of the infection, that this agent reacts specifically with the pneumococci, and that as a result of this reaction the bacteria become susceptible to phagocytosis.

This discussion of pneumococcal pneumonia illustrates in a general manner the course of events in a fairly typical infectious disease, with particular reference to the third line of defense involving the formation during the course of the infection of an *increased* and *specific* resistance of the host to the infectious agent. How did the modern ideas of immunity, an increased resistance of a host to a parasite, originate and develop?

**Development of the Science of Immunology.** Long before it was discovered that bacteria and other microorganisms may cause infectious disease, it was recognized that people vary in their susceptibility, i.e., their resistance to disease. It was also recognized that individuals who had recovered from certain diseases were generally not again susceptible to the same ailment. This was particularly true of infections such as measles, scarlet fever, and smallpox. Centuries ago the Chinese observed



FIG. 20-3. Edward Jenner, discoverer of a method for immunization against smallpox.

that individuals inoculated with material obtained from smallpox lesions generally developed a mild localized infection and became resistant to the more generalized type of infection. Jenner in 1796 observed that English dairymaids who had contracted cowpox (vaccinia), a mild, smallpox-like disease, from cattle were subsequently immune to true smallpox (variola). He inoculated fluid from cowpox blisters into children and noted that they developed a mild localized form of cowpox, at the same time becoming resistant to smallpox. The introduction of vaccination as a preventive measure against a specific disease occurred long before the germ theory of disease had been established, but this early work did suggest that a specific material might be the cause of a particular disease and that the body can develop an increased resistance to the development of the disease.

By 1880 it was recognized that chicken cholera is caused by a bacterium now known as *Pasteurella multocida*. Pasteur observed that this organism did not multiply in sterile urine or yeast water, media favorable for the growth of the anthrax bacillus, while it did multiply in



chicken broth. Reasoning by analogy he concluded that this behavior might be representative of what happens when a harmless microbe enters the body, it being harmless because conditions are not suitable for its growth. He also observed that the smallest drop of a culture of this organism on bits of food was sufficient to produce the disease in the chicken. Guinea pigs, on the other hand, were quite resistant to the organism and developed only a slight abscess at the site of inoculation of the bacteria. However, food contaminated by material from the abscess was highly infectious for chickens and rabbits. This was an early observation of native species immunity and an important contribution to the science of public health, leading to later observations that one species may harbor a parasite with little or no harm to itself and serve as a possible reservoir for the dissemination of the organism to susceptible hosts.

Of still more importance was the observation that chickens inoculated by chance with an old culture of *P. avicidia* developed a mild form of chicken cholera from which they speedily recovered, and at the same time they became highly resistant to subsequent infection with young, virulent cultures of the same organism. Subsequent observations showed that the virulence of this organism gradually decreased as the age of the culture increased, oxygen apparently being involved in the attenuation process. Pasteur then proceeded to develop "vaccines" for the immunization of chickens against chicken cholera. He recognized the similarities in this process of immunization and that earlier introduced against smallpox by Jenner and developed the hope that it would be possible to reduce artificially the virulence of all infectious agents to such an extent that they could be employed for immunization purposes. Pasteur was successful in attenuating both *Bacillus anthracis* by repeated transfers of the organism at 42°C. and the virus of rabies by passage of the virus through rabbits and desiccation of their infected spinal cords for different periods of time, and was able to develop methods of employing these attenuated agents for the purpose of immunizing susceptible animals against the virulent form. In time, other methods for attenuating pathogenic forms were developed, and it was also noted that suspensions of killed bacteria were of value as immunizing agents in some instances. In 1890 Behring and Kitasato observed that animals may be artificially immunized against diphtheria toxin by repeated injections of sublethal doses of the toxin. Immune sera from these animals neutralized diphtheria toxin in the test tube or, when injected into a susceptible animal, would protect it against the subsequent injection of lethal doses of the toxin. This suggested a possible therapeutic use for diphtheria antiserum, and Behring first employed the antiserum (a serum containing neutralizing substances, antibodies, against a specific substance or cells) as a therapeutic measure on Christmas eve, 1891.



In the period 1888 to 1890, Nuttall in England and von Fodor in Germany reported that defibrinated bloods frequently had some bactericidal action which appeared to be destroyed on heating at 60°C. These observations were confirmed in 1889 by Buchner, who regarded the germicidal property of blood as due to the presence of a thermolabile substance which he named *alexin*. Observations of this nature led to the development of the *humoral concept of immunity*, a concept that increased resistance is brought about by protective substances, *anti-*

*bodies*, produced by the body and present in its circulating fluids. The nature of these humoral forces began to be evident with the studies of Pfeiffer in 1894, of Bordet in 1898, and of Ehrlich in the early 1900's. Ehrlich's contributions were primarily the introduction of quantitative methods and the development of theories concerning mechanisms of formation and of reaction of antibodies. His ideas were of considerable importance in the development of immunology, and since they were primarily on mechanisms of reaction, they will not be considered here.



FIG. 20-4. Paul Ehrlich, early student of immunology and chemotherapy.

Pfeiffer, in 1894, observed that guinea pigs which had recovered from experimental cholera were resistant

to subsequent intraperitoneal inoculations of the infectious agent, *Vibrio comma*, and that these bacteria were rapidly destroyed in the peritoneal cavity. Inoculations of the same numbers of bacteria from the same culture into normal guinea pigs resulted in little or no destruction of the vibrios, and the animals contracted cholera. Pfeiffer made three observations fundamental to our modern concepts of immunology: (1) the immunity developed in guinea pigs which recovered from cholera was specific against the cholera vibrio, (2) this antibacterial immunity could be transferred to normal animals inoculated intraperitoneally with immune serum and virulent bacilli (transfer of immunity first observed by Behring and Kitasato with antitoxins), and (3) while either heated or unheated immune serum was effective as a protective agent in vivo, the heated serum was not bactericidal in vitro. This suggested that two agents were involved, one heat-stable and the other heat-labile.

Pfeiffer's studies were confirmed by Bordet in Pasteur's laboratory in 1898. Bordet extended these observations and demonstrated that unheated immune serum would specifically lyse cholera vibrios in hanging-drop preparations while the same serum heated at 56 C. for thirty minutes had no effect other than causing the bacteria to clump together. Addition of fresh normal serum to the latter mixture induced dissolution of the cells. Bordet concluded that heat-stable agents, which exhibit specific combining power for the cells against which they are formed, appear in the blood stream during the course of an infection. These immune bodies (antibodies) react with the bacteria against which they were formed and render the latter susceptible to the bactericidal and lytic power of a heat-sensitive agent, apparently the alexin of Pfeiffer, normally present in blood. Since this agent augments the action of the immune body, Ehrlich proposed the name *complement* as indicative of its action, and this term is commonly employed at the present time.

During the same period other observations on humoral aspects of immunity were made which laid the foundation for the science of immunology, a science closely allied with the field of medical bacteriology. In 1895 Denys and Leclef observed that phagocytosis of a particular species of bacteria was enhanced by the addition of antiserum developed against that organism, while Wright in 1903 demonstrated that the action of the immune serum was directed against the bacteria rather than against the phagocytes. He named the immune body *opsonin*, the word literally meaning to prepare food. In 1896 Gruber and Durham reported that immune serum added to a suspension of the bacteria against which it had been developed caused the cells to clump together, or agglutinate. Widal, in the same year, independently demonstrated that the agglutination reaction could be employed for the diagnosis of typhoid fever, serum from typhoid patients possessing the ability to agglutinate typhoid bacilli. In the following year, 1897, Kraus demonstrated that immune serum reacts specifically either with bacterial cells, causing them to clump together, or with proteins extracted from the same species of bacteria, precipitating the proteins from solution. The nature of the various reactions and reagents mentioned above will be discussed in Chap. 21.

The various observations on the nature of immunity and of the immune reactions around the beginning of this century made evident that both cellular and humoral factors are involved in the defense of the host against a parasite or its deleterious products. The phagocytic system constitutes the second line of defense of the body, while the specifically acquired immune responses constitute a third protective mechanism, are aided at times by complement, and help the phagocytic systems in the disposal of the pathogenic agent. The exact role of the circulating im-

immune bodies in defense of the host is unknown. There is also evidence which suggests that specific local tissue immunity may play a role in the defense of the host.

**Immunization.** We have considered that the third line of defense becomes active during the course of an infection or that it can be stimulated into specific activity by the injection of attenuated or killed organisms or their specific products such as exotoxins. Agents which can be employed for the artificial production of the immune state are popularly called *vaccines*, although strictly speaking the terms vaccine and vaccination refer only to the material and procedure employed in immunization against smallpox. In immunology the term vaccine is commonly employed for suspensions of attenuated or killed organisms employed as immunizing agents. It must be borne in mind that the vaccine or immunizing agent does not of itself produce increased resistance on the part of the host. The material inoculated into the individual animal stimulates the animal to produce for and by itself substances which will neutralize the complex material, ordinarily proteinaceous, of which the immunizing agent is composed. The body itself builds up specific defense substances, *antibodies*, against the pattern presented by the particular immunizing agent. The production of increased resistance, either as a result of having the actual infection or of being inoculated with attenuated material, is spoken of as *active immunization*. Temporary immunization against a number of infectious agents may be accomplished by the injection of antibodies produced by and present in the serum of another animal which had been actively immunized or has had the infection. The injected animal has not itself produced the antibodies involved in this protection, and therefore this means of establishing increased resistance is spoken of as *passive immunization*. Such immunization is temporary in character, as the injected antibodies disappear in a few days or weeks. The antibody-containing serum employed in passive immunization is known as an antiserum and is generally named after the material against which it was produced, as, e.g., diphtheria antiserum or diphtheria antitoxin. Antisera contain neither microorganisms nor their toxins but instead antibodies preformed against these agents. Today children usually are actively immunized against diphtheria with diphtheria toxoid, but a person exposed to diphtheria can be passively immunized with antiserum as a protective measure.

Passive immunization is of value in the attempted prevention of the development of certain infectious diseases after exposure to the infectious agent. The results of passive immunization on the whole have not been too encouraging. Antisera have been employed with success as therapeutic agents, particularly in the treatment of diphtheria, pneumococcal



pneumonia, and epidemic meningitis, and have proved to be of some value in the prevention or treatment of tetanus, gas gangrene, botulism, measles, and certain streptococcal infections. Antibodies are present in the gamma globulin fraction of serum, and gamma globulins are often employed instead of whole serum.

Active immunization, vaccination in the popular sense, is most successful against those agents responsible for infections which leave a lasting immunity. It is of less value for general use against infectious agents which do not elicit the establishment of a lasting immunity in the host, although it may be worth while against infections such as influenza when these appear in epidemic form. Information is scarce as to why certain infections are followed by relatively permanent immunity, others are followed by only a temporary period of increased resistance, and still others by no appreciable change in resistance against the specific etiological agent. In the case of diseases of man, the use of vaccines is relatively limited, much to the disappointment of early workers in immunology who predicted that it might be possible to immunize against the majority or all of the common infectious diseases. It should be remembered that immunity is a relative condition and not an absolute one, the immunized individual frequently possessing a resistance greater than that of the nonimmunized person. Immunization is extremely valuable against smallpox, diphtheria, and tetanus and should be employed against typhoid, cholera, influenza, yellow fever, typhus fever, poliomyelitis, and certain other less common infections in areas where these diseases are present in endemic or epidemic form. It should be remembered that immunity is not established at once following the one or more injections of the immunizing agent, since time is required for antibodies to be formed in suitable concentrations. As a rule, active immunization is of no value as a therapeutic measure and should be initiated before exposure occurs. An apparent exception is rabies, against which immunization is initiated after exposure has occurred. Actually immunization against rabies is a prophylactic measure which takes advantage of the fact that considerable time may be required for the virus to pass from the area contaminated by the bite of a rabid animal to the brain and spinal cord. Immunity is generally established between the time that the individual is contaminated with the virus and before the general infection can be established.

**Types of Immunity.** We have seen that immunity can be classified into two general types, natural immunity and acquired immunity. Natural immunity is that resistance or nonsusceptibility normally possessed by inheritance and is independent of antibodies, being based on the failure of certain biological groups to serve as suitable hosts for microorganisms pathogenic for other groups. The extent of natural immunity may vary



from species to species, may vary among races of the same species, and may vary from individual to individual or in the same individual. Acquired immunity may be subdivided into naturally acquired immunity and artificially acquired immunity. Naturally acquired immunity is that resistance developed as a result of having had a particular infection. Artificially acquired immunity is established either actively, following the use of attenuated or killed organisms or their exotoxins, or passively, by the injection of antibodies. The immunity against various infections which is possessed by infants might appear to be of the naturally acquired type but is actually an example of passively acquired immunity, antibodies passing from the mother to the developing fetus. Immunology, strictly speaking, cannot be considered a part of the subject matter of general bacteriology since it deals to a great extent with the reactions of a host rather than of the parasite. However, the nature of the microbe or its products plays such an important part in the eliciting of the immune response that space was devoted to a consideration of the general principles of this science. Moreover, studies of the reactions between bacteria, or their constituents, and antibodies formed against them have contributed markedly to knowledge of the antigenic composition of bacteria. These reactions, commonly considered under the general term of serology, will be considered in the following chapter.

**Hypersensitivity.** We have been considering the antigen-antibody reactions as protective mechanisms, but we now turn to an immunological paradox, allergy or hypersensitivity. The injection of an antigen may cause an animal to become sensitive (hypersensitive) to that antigen, and on subsequent injection of the same antigen, no matter how innocuous it may be, the animal may react as though a violent poison had been injected.

In the earlier days of antitoxin therapy, in a few instances the patient suddenly collapsed or died following injection of the antitoxin. Investigation of this phenomenon proved that it was independent of the antitoxin content of the serum and was due to a peculiar sensitivity of the individual to the normal serum proteins which were injected along with the antitoxin. With improved methods for the purification of antitoxin such untoward reactions have become very rare.

Studies on the mechanism of this reaction brought attention to a wide variety of different, but closely related phenomena. Knowledge of these reactions is incomplete and is hampered by a confused terminology. The terminology employed here is representative rather than what might be considered best by many workers. The term allergy means altered reaction and is used more or less synonymously with hypersensitivity. Under this general term are included at least five different types of altered reac-

tivity (as compared with the response of a normal individual): atopy, the idiosyncrasies, anaphylaxis, serum sickness, and allergy of infection.

By atopy is meant an apparently hereditary, or at least inborn, tendency toward hypersensitivity to certain substances, a state which cannot be produced experimentally. Hay fever, rose fever, and susceptibility to many pollens appear to be such conditions. Asthma is another atopic condition generally caused by animal danders, mold spores, and other proteinaceous materials floating in the air. Hypersensitivity to food, resulting in digestive disturbances and hives following the ingestion of the food-stuff to which the individual is sensitive forms a third type of atopic hypersensitivity. Poisoning induced by the toxic principles of poison oak or poison ivy and the violent response of some individuals to medicinal doses of a particular drug are classed under the idiosyncrasies, rather than as atopies, along with idiosyncrasies to particular foods, if these altered reactions are the result of exposure to the agent and if inherited susceptibilities are not involved in the individual's response to the agent. Information concerning these types of hypersensitivity is rather confusing and, since it has little to do with the bacteria, will not be discussed further.

**Anaphylaxis.** When one injects a guinea pig with a minute amount of an antigen such as egg white and makes a second injection about two weeks later, this second injection will immediately be followed by marked reaction on the part of the pig and usually results in sudden death of the animal, an example of true anaphylaxis. The essential requirements for the production of anaphylaxis or anaphylactic shock are a preliminary injection of a *sensitizing* dose of the antigen, which during a period of incubation elicits the formation of the hypersensitive state, and the injection of a second dose of the same antigen at a later date into the sensitized animal. The symptoms of anaphylaxis are the same regardless of the substance employed to induce the shock, but the reaction is as specific as any of the antigen-antibody reactions.

Other animals may show similar anaphylactic reactions although the symptoms vary from species to species, since the reaction appears to take place primarily in collections of involuntary or smooth muscle, the location of the most reactive muscles being different in different animal species. The sensitized state may also be induced by passive sensitization, i.e., the injection of serum from a hypersensitive animal into a normal animal. Considerable evidence points to the probability that anaphylaxis is the result of an antigen-antibody reaction generally occurring in the tissues rather than in the blood stream, and that histamine, or histamine-like substances, are liberated as a result of the reaction. The symptoms of anaphylactic shock are ascribed to the liberation of this substance or substances within the tissues. The same concept holds for animals that

are passively sensitized, it being assumed that the antibody is taken up by tissue cells. Individuals who exhibit true hypersensitivity of this character can generally be desensitized by repeated, frequent subcutaneous injections of minute amounts of the antigen.

Those occasional cases of death following the second administration of an antitoxin or antibacterial serum prepared in a horse or other animal were probably an anaphylactic response, the individual apparently having been sensitized by the original injection of the animal serum. There occurs more frequently another type of reaction called *serum sickness*, which fortunately is much less serious although it may be highly uncomfortable to the individual. It appears not immediately, but about ten days after the injection of the antiserum, and the symptoms are those of a fever together with an itchy rash, hives. The incubation period for the development of serum sickness is about the same as for the development of anaphylactic sensitization, and the two phenomena appear to be similar but not identical with each other.

**Allergy of Infection.** By allergy of infection is meant the hypersensitivity induced as a result of having had a particular infection. When tuberculin, an extract prepared from old cultures of the tubercle bacillus, is injected into a tuberculous guinea pig a reaction unlike anaphylaxis occurs, the animal after a few hours becoming ill and growing progressively weaker until, if the dose is large enough, death results, generally in 12 to 48 hours. No reaction is noted when tuberculin is injected into a normal guinea pig, showing that the tubercular animal had been sensitized during the growth of the tubercle bacilli in the animal. Marked reaction to tuberculin may also be noted in individuals who have, or have had, tuberculosis. The *tuberculin test* is based on this phenomenon, but in order to prevent untoward accidents, the tuberculin is injected only into the superficial layers of the skin and only in minute amount. Results of the tuberculin test are conclusive (with a few minor exceptions) only when the test is negative, since a positive test may result in people in which the disease is no longer active. The majority of adults in metropolitan areas are tuberculin-positive, and many of these positive reactions may be due to repeated exposure to tubercle bacilli with resultant sensitization. Similar skin tests are employed in the diagnosis of other infectious diseases or in the determination of the pollens or other proteins to which an individual is sensitive. Detailed discussions can be found in larger texts or specialized monographs. Much remains to be learned about the nature and mechanisms of the different types of altered reactivity.

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## CHAPTER 21

### THE SEROLOGICAL REACTIONS

The early studies in immunology indicated that antibodies are formed against cells or proteins foreign to the animal body when these substances gain entrance to the circulating fluids. The material inducing antibody formation against itself need not be pathogenic or toxic to the animal. Therefore, antibody formation is not a line of defense against pathogenic agents only but is actually a mechanism of broader biological significance.

An animal may ingest a *protein* such as egg white, and this material is converted in the digestive system into materials capable of being assimilated by the cells comprising the body of the animal. The amino acids or other products of protein digestion are distributed in the body by means of the circulating fluids. Egg white itself is not a suitable food for the individual cells, since they lack the appropriate digestive enzymes, and it therefore would serve no useful purpose in the circulating fluids. Furthermore it is such a large molecule that it would not be readily removed from the blood by the kidneys, and at the same time it appears to be too small for effective removal by the phagocytes. When *injected* into the tissues or circulating fluids, it is a material foreign to them and should be eliminated. Certain tissue cells respond to the presence of this foreign material by forming antibodies directed against the egg white. It can be demonstrated in the test tube that the antibody reacts specifically with egg white to form complexes of egg white and antibody which settle out, precipitate, from solution. The nature of the reaction in the animal body is unknown, but there is some evidence which suggests that complexes are formed which can be removed by the phagocytes.

Any substance which, when introduced into the blood or tissues of an animal, elicits the formation of antibodies is called an *antigen*. Conversely, any substance which is formed as a result of the injection of an antigen and which will react with that antigen in a specific manner is called an *antibody*. Any complete protein foreign to the animal body and soluble in body fluids will act as an antigen, and there are certain complex lipoids and polysaccharides which also act as antigens, either by themselves or, as some workers suggest, after self-coupling with a protein which need not be foreign to the body.

The most important or interesting attribute of antigens and antibodies is their extreme specificity. This means that an antibody will react only with the antigen against which it was formed or with an antigen possessing very similar chemical groupings on its surface. Landsteiner and others have demonstrated that the specificity of a protein may be altered by a variety of methods, particularly by the addition of various chemical groupings to the protein molecule. An antigen thus appears to be a very complex molecule, generally proteinaceous, whose antigenic specificity is controlled by the presence of either naturally occurring or artificially added surface groups. To illustrate this concept, let us consider a protein such as egg albumin chemically linked with molecules such as tartaric acid. Antibodies formed against the egg albumin-tartrate complex will precipitate it, but they will not react with and precipitate egg albumin itself. On the other hand, these same antibodies may react with any protein-tartrate complex, their specificity being directed primarily against the tartrate groups (even against an optical isomer) on the protein molecule and not against the protein itself. When the antibody is mixed with tartaric acid alone, no demonstrable reaction is observed, but if the protein-tartrate complex is added later, no precipitate forms. This indicates that the antibody can react with tartaric acid alone. The reactivity of the antibody is neutralized as a result of the reaction, but the antibody-tartrate complex is of such a nature that no precipitate is formed, demonstrable antigen-antibody reactions generally being observed only when both antigen and antibody are relatively large molecules. Chemical groups which impart specificity to the antigen, which can be split off from the antigen, and which can by themselves react with antibodies but are unable to elicit the formation of antibodies are known as *partial antigens*, or *haptens*.

It was originally believed that a single species of bacterium contained, or acted as, a single antigen. More recent studies show that a bacterium does not always react as a single antigen and that it actually contains more than one antigen, each usually specifically located in the cell. For example, the antigenic type specificity of different types of pneumococci is determined by the chemical nature of the capsules of the smooth, virulent forms. The avirulent or rough forms are variants which do not possess a capsule and are then characterized by species rather than by type specificity, all pneumococci possessing the same cellular protein constituents. Thus, the pneumococci possess a common antigen or antigens in the body of their cells and type-specific haptens or antigens in their capsules. Antisera, to be of value as therapeutic agents, would have to contain antibodies against the capsular antigens. Similar complexity of antigenic composition and structure is noted with many bacteria. Motile cells of organisms such as *Salmonella typhosa* possess an antigen (*H* anti-

gent in their flagella which is different from the antigens on the surface and in the body of the cells. Capsular and flagellar antigens are known as *surface antigens*, while those within the cell are designed as *cell-body*, *somatic*, or *O antigens*. There is evidence which suggests that nonencapsulated, nonflagellated species of bacteria may have surface antigens which are different from their somatic antigens.

The antigenic structure of the virulent form of an organism must be considered in the preparation of vaccines against that organism. Immunization against the somatic antigens of an organism such as *S. typhosa* in the avirulent rough form would not necessarily induce increased resistance against the virulent, flagellated form. Also changes in virulence and antigenicity may result when such an organism undergoes dissociation, without necessarily losing its flagella at the same time. This complexity of the antigenic structure of living organisms must be taken into account in the attempted control of the pathogenic forms by means of immune substances, antibodies. To confuse the situation further, the antibody titer (concentration as determined by highest dilution active in producing a demonstrable change in vitro) does not always correlate with actual immunity as determined by animal tests. Furthermore, a high degree of resistance may be established against one organism but little or none against other infectious agents. More information is needed concerning the third line of defense. Analyses of the antigenic composition of bacteria are also of value in that they indicate the complex chemical composition of bacteria and also taxonomic relationships between different species.

The nature of antibodies is unknown except that they are proteins and can be removed from the serum by precipitation with the aid of chemical agents which precipitate that type of protein molecule known as globulin. Antibodies formed against the same protein, but by different animal species, appear to be chemically different proteins but probably contain the same reacting groups, since they all react with the same substance in a similar manner. However, the antibody protein must as a whole be a protein characteristic of the animal in which it was formed, else it would be not-self material.

**Theories of Serological Reactions.** Two general theories are supported at the present time concerning the nature of the reaction between an antigen and its antibody. According to one school of thought, antigen and antibody combine in stoichiometrical proportions much as simple molecules react with each other. Both the antigen and the antibody may have more than one reactive group per molecule. Therefore, an antigen-antibody complex can react with additional molecules of either antigen or antibody, or both, and in that manner large unstable complexes are



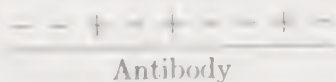
formed which readily settle out of solution or suspension. The other point of view is that the antigen is adsorbed by the antibody, or vice versa, and that the reaction does not follow the classical laws of chemistry but instead is a colloidal chemical phenomenon in which the union follows not the law of definite proportions but rather the proportions of typical adsorption reactions.

In considering the mechanism of the serological reactions, it is apparent that we must differentiate between the *union* of antigen with antibody and the occurrence of a *demonstrable change* subsequent to the antigen-antibody union. Bacterial cells will unite with their antibody in the absence of electrolytes, but agglutination generally cannot be observed under these conditions. Agglutination will occur in a short period of time after the addition of salt to such an antigen-antibody mixture. Or, antigen and antibody will unite in the absence of complement, and complement will be fixed if added at a later time to the original mixture. In all cases the reaction occurs in two stages: first a primary union between antigen and antibody and then secondary demonstrable changes, such as precipitation of proteins from solution, agglutination of cells, or lysis of susceptible cells by complement. However, the two stages in the reaction may occur concurrently.

The exact nature of antigens, of antibodies, and of the reactions between these substances is far from being completely understood. However, we can attempt to develop a simplified general concept of the basic nature of these agents and their reaction with each other, bearing in mind that we are dealing to some extent with ideas rather than facts. First we may picture both antigens and antibodies as complex protein molecules, each possessing a variety of chemical groupings on the surfaces of the molecules. There would be a characteristic distribution of positive and negative charges, or electrical fields of force, and of chemical groups over the surface of an antigen molecule, which can be schematically illustrated as



If combination of antigen and antibody occurs as a result of the neutralization of charges or fields of force, it could be conceived that antibodies are globulins with surface groups molded during their formation "to fit" their antigen, both in surface structure and electrostatic forces. This would give a surface which might be represented as





It is apparent that antigen and antibody would be complementary to each other (compared to lock and key in early studies). Antigen and antibody could unite in the following manner:

Antigen									
+	+	-	+	-	+	+	-	+	
-	-	+	-	+	-	-	+	-	
Antibody									

and neutralization of the electrostatic forces at the combining sites would occur. As a corollary of this idea, antigens (or antibodies) having practically the same reactive groups or surface forces might unite with the same antibody (or antigen). Since an antigen or antibody can have more than one combining group, opportunity for combination with more antigen or antibody is available, and three-dimensional complexes can be formed. Haptens could react with antibodies in the same manner, but no visible reaction would be observed since the hapten contains only one combining group. Marrack and Heidelberger have postulated that antigens and antibodies react to form a lattice-like structure, the final product of the reaction between these two agents depending on the relative amount of each substance. This may be illustrated for multivalent antigens and antibodies in a two-dimensional way as in Fig. 21-1. Precipitation or agglutination would be observed only in mixtures in which neither reagent was present in marked excess. Electrolytes may influence the original union between antigens and antibodies and/or the association of these complexes to form microscopic clumps large enough to settle out of solution or suspension. The antigen-antibody complex has surface forces so different from either of the original reactants that the complex is readily ingested by phagocytes or binds complement when the latter is present. Union of antigen and antibody, if the former is not present in marked

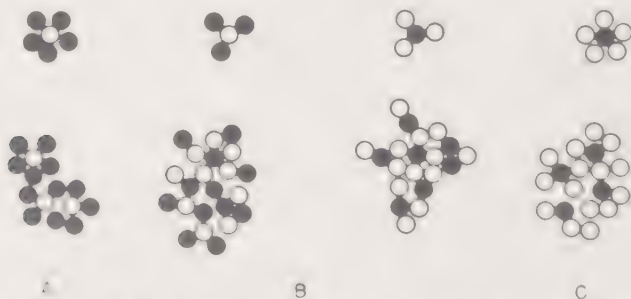


FIG. 21-1. Illustration of the lattice-structure hypothesis of antigen-antibody union, based on the studies of Marrack and Heidelberger. ○ represents antigen, ● antibody; A, antigen in excess; C, antigen in excess; and B, intermediate concentrations of both reagents.

excess, would also tend to neutralize the toxicity of antigens such as toxins and to form microscopic floccules or macroscopic precipitates.

**The Unitarian Hypothesis.** The early workers in the field of immunology considered that the different antigen-antibody reactions were due to the presence in immune sera of separate, distinct antibodies, and therefore names were given to indicate the particular reaction induced by each antibody postulated to be formed against one antigen. These names are listed in Table 21-1 together with the material eliciting the formation of the

TABLE 21-1

Antigen	Antibody	Type of reaction
Protein	Precipitin	Precipitation
Toxin	Antitoxin	Neutralization of toxicity
Virus	Antiviral	Neutralization of infectivity
Bacteria	Agglutinin	Agglutination
Bacteria	Opsonin	Increased phagocytosis
Bacteria	Bactericidin	Bactericidal
Bacteria	Lysin	Lysis

antibody and the reaction observed directly or indirectly after mixing the antigen with its antibody. These reactions are schematically illustrated in Fig. 21-2.

The unitarian concept, now generally accepted, implies that there is no multiplicity of antibodies as listed above and that instead these names indicate different activities of but a single antibody formed against a specific antigen. It should be emphasized that a different antibody would be formed against each antigen of a bacterial cell and that different reactions are observed when the tests are carried out under different conditions. To illustrate the unitarian hypothesis, let us consider the reactions between a bacterial antigen and its antibody. The bacterium would be killed by the antibody (bactericidin) aided by complement, and lysis of susceptible cells would follow. When lysis is observed, the antibody would be termed a lysin. In the presence of phagocytes, the antibody (opsonin) would enhance phagocytosis. And finally the antibody (agglutinin) would induce agglutination of intact cells, or it (precipitin) would precipitate the cellular protein when the cells or cellular antigen molecules, respectively, are in suspension or in solution in saline. When toxins or viruses are employed as antigens, the reaction most commonly observed is neutralization of toxicity or infectivity as determined by animal experimentation. When bacteria are employed as the antigenic material, a different antibody would be formed against each antigen comprising

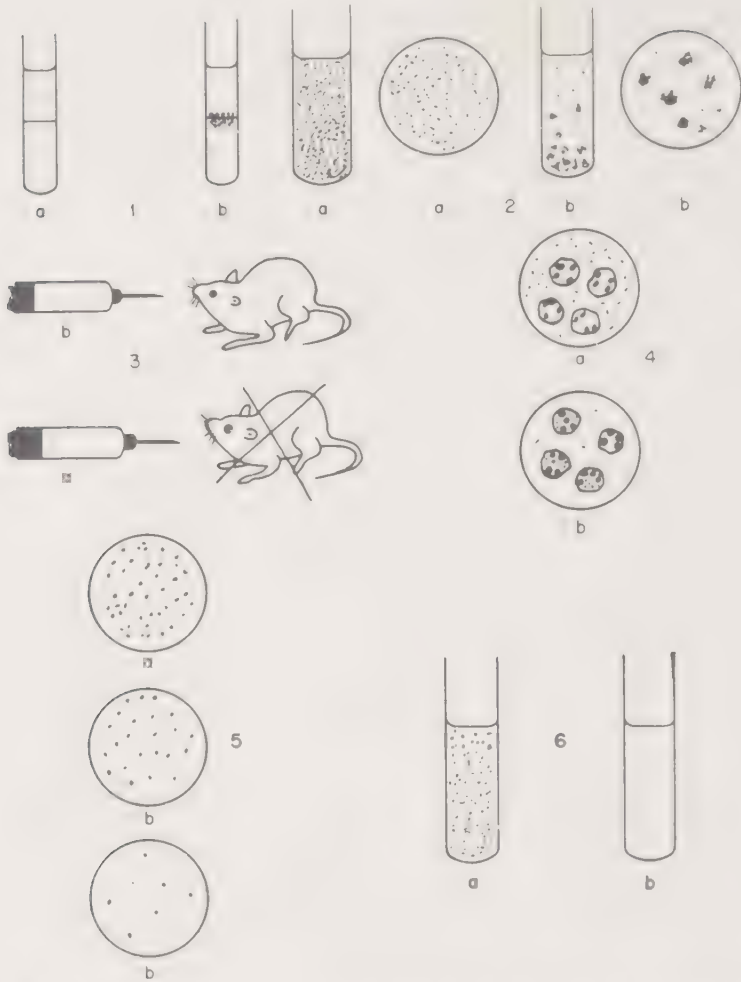


Fig. 21-2 Schematic representations of the results of different serological procedures. Control tests with normal serum are labeled (a), the results with immune sera (b). (Sera heated at 56°C. to inactivate complement.)

1. Precipitin test; precipitate formed at junction of immune serum and protein solution.
2. Agglutination tests; macroscopic and microscopic agglutination of bacteria illustrated.
3. Neutralization tests; toxin, or virus, neutralized by its antibody as indicated by animal tests.
4. Phagocytosis; little or no phagocytosis in the absence of immune serum.
5. Bactericidal tests; upper plate count represents initial number of bacteria; middle after exposure to immune serum; and lower after same exposure to immune serum and complement.
6. Hemolysis; red blood cells lysed in the presence of both immune serum and complement; a in this example representing either RBC + normal serum alone or + normal serum and complement.

the cell, but in ordinary *in vitro* tests only the surface antigen or antigens need react with the corresponding antibody. There are some apparent exceptions to the unitarian hypothesis, but these are of too technical a nature to consider here. Since it is convenient to study the individual types of reaction, the various names indicating the different types of activity of the same antibody are still employed.

#### APPLICATIONS OF THE ANTIGEN-ANTIBODY REACTIONS

The specificity of the antigen-antibody reactions and the rapidity with which they can be carried out make them valuable tools in the diagnosis of infectious diseases and for the identification of various bacteria and proteins, or even relatively simple molecules which can act as haptens. As far as their application to the diagnosis of infectious disease is concerned, the reactions can be employed for the identification of either bacteria or antibodies against them, using a known antiserum or known bacterium, respectively.

**The Precipitin Reaction.** When an animal is given a series of injections of a protein foreign to the species, antibodies will be formed and can generally be demonstrated in the blood within 10 days after the first injection. The concentration of the antibody and in some instances its reactivity increase as the injections are continued, reaching a maximum value dependent on the nature of the antigen and of the immunized animal. When serum from the immunized animal is mixed with the antigen, definite floccules or precipitates composed of antigen and antibody are formed. The reaction is a very sensitive one and can be employed to detect the presence of extremely minute amounts of the protein employed as the antigen. The sensitivity of the test is further increased when the solution of antigen is carefully layered over the antiserum in a narrow test tube, precipitation taking place in the zone of demarcation of the two solutions. The reaction is highly specific and can be employed to differentiate between closely related proteins which cannot be distinguished from each other by ordinary chemical tests.

One of the more important applications of the precipitin test is in the medicolegal identification of blood stains. The serum of a rabbit strongly immunized against human blood will form a precipitate with human blood in dilutions as high as 1:100,000 but not with other bloods except from some of the higher apes. The reaction will detect blood proteins in saline extracts of blood stains, even after the blood has been dry for many years. The reaction is actually one for human proteins, and physical or chemical tests would have to be employed in conjunction with the precipitin test for the final identification of the presence of human blood in the stain.



The precipitin test can also be employed for the detection of adulteration of meat products with other proteins, for the identification of type-specific pneumococcal polysaccharides in the urine or pneumonic sputum of individuals ill with pneumonia, for the detection of other bacterial antigens or haptens, and for the detection or identification of proteins in general or of antibodies against the proteins. The latter application is most commonly employed in detection of the syphilitic state by means of the Kahn test, or modifications of it.

**The Kahn Test.** The Kahn test employs a suitably prepared alcohol-ether extract of beef heart as the antigen since this contains a material which, by some quirk of nature, reacts in a manner analogous to that which would be exhibited by *Treponema pallidum* if it could be cultivated in the laboratory and used directly as the antigen. A precipitate forms when a properly prepared suspension of this "antigen" is mixed with serum from syphilitic individuals, while no precipitation occurs on mixture with serum from noninfected individuals. Although the test is not entirely specific for syphilis, results of tests properly carried out are reliable with minor exceptions for the identification of the syphilitic state. The actual interpretation of results of the Kahn and other precipitation tests, as well as of the Wassermann test, for syphilis requires a broader background than can be developed here.

**Neutralization of Toxicity.** The bacterial exotoxins are proteinaceous in character and act as antigens in the body. Toxin and antitoxin concentrations were originally determined by toxicity and neutralization tests employing susceptible animals. Nicolle in 1919 observed that toxin and antitoxin will react in vitro with the production of a precipitate if the reactants are mixed in appropriate concentrations. Ramon developed this precipitation test into one which can be employed for the quantitative titration of either a toxin or an antitoxin, when the concentration of one reactant is known. This test is of considerable value since it can be carried out in a short period of time and greatly reduces the number of animals required for the final standardization of toxin or antitoxin.

More than flocculation or precipitation is involved in the toxin-antitoxin reaction. The reaction can occur with concentrations too small to form a visible precipitate; yet a lethal dose of toxin becomes nontoxic on admixture with antitoxin in equivalent amounts. The toxin is not destroyed and by various means (such as dilution) can be set free, i.e., the reaction is a reversible one. In some manner, toxicity is masked by the antitoxin, and in time the toxin does appear to undergo change with actual loss of toxicity, the reaction no longer being reversible. Neutralization of toxin by antitoxin has been employed since Behring and Knasato's studies in 1891 for the specific treatment of individuals ill with diphtheria or tetanus. The neutralization of the infectivity of a virus may be analo-

gous to the neutralization of a toxin; at least the two types of antigen-antibody reactions have a number of characteristics in common.

**The Agglutination Reaction.** The agglutination reaction is very similar to the precipitation reaction with the exceptions that the antigen or antigens are bound in a large complex rather than being in solution and the entire cell rather than the antigen alone is involved in the clumping process. It was mentioned that an agglutination test was first employed in 1896 by Widal in the diagnosis of typhoid fever. The Widal test is based on the fact that antibodies against *Salmonella typhosa* appear in the blood about a week after the onset of the infection. It was at first believed to be a highly specific test, but subsequent observations indicated that the same serum might agglutinate other, ordinarily closely related, species of bacteria. In general, the test is diagnostic if the serum in a dilution of 1:80 or higher agglutinates *S. typhosa*, provided that the individual had not been immunized against this organism so as to possess typhoid antibodies already. Are the antigen-antibody reactions highly specific, and if so, what is responsible for the cross-agglutination reactions?

When a rabbit is immunized by repeated injections of typhoid bacilli, a high concentration of antibodies develops in the blood stream. Agglutinin concentration is ordinarily expressed in terms of the highest dilution of the antiserum which will produce agglutination of a standard suspension of the cells employed as the antigen, and a titer of 1:10,000 represents a very active antiserum. When such an antityphoid serum is not highly diluted, it frequently brings about the agglutination of closely related species of bacteria and results such as the following might be obtained:

<i>Bacilli agglutinated</i>	<i>Antiserum diluted</i>
Typhoid bacilli .....	1:10,000
Dysentery bacilli .....	1:500
Colon bacilli .....	1:100

Results of this nature at first cast doubt on the specificity of antigen-antibody reactions. It was later demonstrated that cells of different, but closely related, species may contain one or more antigens in common, but not necessarily in the same concentrations or the same locations in the cell, and therefore an antiserum may react with closely related species of bacteria or with proteins of similar structure.

For example, let us assume that the three organisms mentioned above each contain four antigens as listed in the following hypothetical table:

Typhoid bacilli .....	<i>A, B, C, D</i>
Dysentery bacilli .....	<i>E, F, C, G</i>
Colon bacilli .....	<i>H, I, J, D</i>

Also let us assume that the concentrations or reactivities in stimulating antibody production of the different antigens decrease from left to right

as listed above. Immunization with typhoid bacilli would elicit the production of antibodies *a*, *b*, *c*, and *d*, and this antiserum would strongly agglutinate typhoid bacteria. Since it contains a small amount (or low reactivity) of antibody *c*, the serum in high concentration would bring about the agglutination of dysentery cells. Antibody *d* is present in very small amount, and hence agglutination of coli is observed in dilutions of the antiserum no greater than 1:100. Thus we see that species specificity is explainable on the basis that each species of bacterium (or of blood proteins, etc.) contains one or more dominant proteins or haptens which are characteristic of that particular bacterium, while the group reactions can be explained on the assumption that certain less dominant antigens are common to a group of closely related species.

A culture suspected of being *S. typhosa*, isolated from an individual supposedly having typhoid fever, could be identified by the fact that it was agglutinated by a high dilution of an antityphoid serum. But there might be some doubt as to the specificity of the test. It could be made more specific by first mixing the antiserum with suspensions of dysentery and colon bacilli. Antibodies *c* and *d* would react with the dysentery and colon organisms and could be removed from the antiserum, together with the cells, by centrifugation. This would leave antibodies *a* and *b* in the antiserum, and hence the only cells with which they would react would be *S. typhosa*. Agglutination of the cells from the culture by a dilution of the purified antiserum would then be highly specific for *S. typhosa* alone. Many such purified and highly specific antisera have been prepared by means of this technique, known as *antibody adsorption*. These purified sera are of extreme value in the identification of antigens and the determination of the antigenic structure of microorganisms.

The agglutination test for the detection of antibodies in a patient's serum can be carried out on a macroscopic scale in the test tube or on a microscopic scale in a drop of serum and of known organisms. In addition to being of value for the detection of antibodies against *S. typhosa* during the course of typhoid fever, the test can also be employed for the diagnosis of paratyphoid fevers, Asiatic cholera (*Vibrio comma*), undulant fever (*Brucella abortus*), tularemia (*Pasteurella tularensis*), whooping cough (*Hemophilus pertussis*), typhus fever (with the X-19 strain of *Proteus vulgaris* as the antigen), cerebrospinal meningitis (*Neisseria meningitidis*), and occasionally a few other infections. In many instances, it is desirable or necessary to adsorb other antibodies by means of the agglutinin adsorption technique to rule out cross agglutination reactions.

The reverse test to the one just mentioned, one in which a known antiserum is employed for the identification of an unknown bacterium or for checking the identity of a bacterium, is commonly employed in the laboratory. It is of great value in identifying closely related species and types



within a species. Considerable care must be employed in carrying out agglutination tests; the use of purified (adsorbed) antiserum is to be recommended, and proper controls must always be included. The various technical factors involved in the reactions are outside the scope of this book.

Another interesting application of the agglutination reaction is the one employed in the determination of blood groups. While this test does not involve microorganisms, it is of general interest in a discussion of agglutination. Earlier attempts at transfusion of blood from one person to another frequently resulted in severe reactions in, or death of, the transfused individual, the donor's red blood cells being agglutinated by the recipient with consequent blocking of circulation of the blood. Landsteiner in 1900 reported the presence of two distinct antigens and of two corresponding antibodies in various samples of human blood. Landsteiner assumed, and it has been found that there are only minor exceptions, that an agglutinogen (antigen) and an antibody (isoagglutinin, since it reacts with an antigen from the same species) against it cannot coexist in the blood of a given individual. His findings were soon confirmed and extended, and at the present time four distinct major blood groups, with a number of minor subgroups or variations, are recognized. Some confusion exists in the terminology of these groups, as they were classified independently by two men at about the same time. The Moss classification has been employed in the United States, but the use of a newer classification, the International or Landsteiner, based on the antigenic structure of the cells, is preferred to prevent confusion. Table 21-2 shows the correlation between the three systems of nomenclature, the isoantigens in the

TABLE 21-2. THE BLOOD GROUPS

Cells			Sera			
Moss			4	2	3	1
Jansky			I	II	III	IV
International			$\alpha\beta$	$\beta$	$\alpha$	0
4	I	O	—	—	—	—
2	II	A	+	—	+	—
3	III	B	+	+	—	—
1	IV	AB	+	+	+	—



cells, the isoantibodies in the serum, and the reaction observed on mixing any one serum with cells. Plus signs indicate agglutination of the cells. The International System of nomenclature is represented by Roman letters, which indicate the isoantigenic structure of the cells, while the Greek letters represent the corresponding isoantibodies.

Individuals of group O are considered universal donors since their red blood cells do not contain any isoantigens and hence are not agglutinated when introduced into a recipient. Since the donor's serum is diluted during the transfusion process by the recipient's blood, the isoantibodies present in the donor's serum are diluted to such an extent that they generally do not elicit agglutination of the recipient's red blood cells. Individuals in group AB are regarded as universal recipients since their serum is free from isoagglutinogens and therefore incapable of agglutinating cells from any donor. Since there are some minor variations in the groups and since other complications are at times encountered, it is wise to transfuse between members of the same group and advisable to match the bloods *in vitro* to see if any reaction occurs before the transfusion is commenced.

The antigens and antibodies upon which the blood groups are based are inherited according to Mendelian principles, and the antibodies are not the result of exposure to a true antigen. Approximately 45 per cent of the population belongs to group O, 40 to group A, 12 to group B and about 5 per cent to group AB. Approximately 85 per cent of the population possess another agglutinogen, known as the Rh factor, in their red blood cells. Transfusion of Rh-positive blood to Rh-negative individuals may induce antibody formation which could bring about agglutination and hemolysis of red blood cells introduced in a later transfusion. Also an Rh-negative mother may be immunized during pregnancy against the Rh factor if the father is Rh positive, giving rise to a fatal disease in the infant known as erythroblastosis foetalis. Other more complicated factors are also involved in the production of this condition, since not all Rh-positive Rh-negative matings give rise to erythroblastosis foetalis, but the factors are technical in character and cannot be considered here.

**Hemagglutination.** Another agglutination reaction, but one that does not involve antibodies as the agglutinating agent, can be observed when certain viruses (e.g., influenza, mumps, smallpox, and encephalitis) are mixed with red blood cells from particular animals. The viruses of influenza can cause a clumping or hemagglutination of chicken or human red blood cells when suspensions of the two are mixed. This is not an antigen-antibody reaction but is induced by the ability of the virus particles to unite with the erythrocytes. This reaction can be specifically inhibited by antibodies against the viral particles. This hemagglutina-

tion-inhibition reaction induced by antibodies serves as a basis for the detection of viral antibodies in immunized people or animals or in individuals suspected of having, or having had, a particular virus disease. In testing serum from a patient suspected of having a viral infection, at least two samples of blood must be obtained, one early in the course of the disease and another after ten to fourteen days. A fourfold or greater increase in titer (highest dilution of the serum preventing agglutination of red blood cells) is considered diagnostic, since antibodies against some of the viruses may be present in low titer in serum from apparently normal individuals.

**Complement Fixation.** Antitoxins, precipitins, and agglutinins are relatively stable substances and will withstand heating for  $1\frac{1}{2}$  hr. at  $56^{\circ}\text{C}$ . On the other hand the lytic activity and the bactericidal action of an immune serum are lost when the serum is allowed to stand for some time or is heated for a few minutes at  $56^{\circ}\text{C}$ , while the ability to enhance phagocytosis may be decreased. This difference in apparent sensitivity of antibodies casts some doubt on the unitarian hypothesis, but it was observed that the antibody was not inactivated and that instead a second component of serum was essential for the activity of these "sensitive" antibodies. The addition of a little fresh immune serum or even of fresh normal serum results in the restoration of activity. This substance, or complex of substances, which complements the activity of the antibody in certain antigen-antibody reactions is called *complement*, or *alexin*, and is a normal constituent of serum, its concentration not being increased as a result of an immunization procedure. The lytic activity of complement is best illustrated with red blood cells as these are relatively fragile and quite susceptible to lysis. The same type of reaction may be demonstrated with many bacteria, particularly the gram-negative ones such as the cholera vibrio, which are susceptible to the lytic activity of complement after the bacteria have united with antibody against them.

When, for example, one immunizes a rabbit against sheep red blood cells, lytic antibodies called hemolysins appear in the blood stream. Following the addition of this antiserum to a sample of washed sheep red blood cells suspended in saline solution, the turbid suspension will clear, and the liquid assumes the bright red color of hemoglobin. This shows that the cells have undergone lysis with the liberation of the hemoglobin which they originally contained. No lysis is observed when one employs heated immune serum because heating destroys complement, but lysis will be observed following the addition of either fresh immune serum or of fresh serum from normal rabbits, sheep, guinea pigs, or other animals. The blood of the guinea pig is particularly rich in complement. This lytic test may be illustrated by the following reactions:

Red blood cells (RBC) + fresh normal serum.....	No effect
RBC + heated normal serum.....	No effect
RBC + heated RBC antiserum.....	No effect *
RBC + fresh, unheated RBC antiserum.....	Lysis of RBC
RBC + heated RBC antiserum + complement in fresh serum.....	Lysis of RBC
RBC + heated RBC antiserum + heated normal serum.....	No effect *

\* Agglutination of RBC may be observed if antiserum is employed in concentrations greater than sufficient to demonstrate complement fixation.

It has been found that complement will unite with any antigen-antibody complex without necessarily producing any detectable change in test-tube experiments in agglutination, precipitation, or toxin-neutralization experiments. Phagocytosis may frequently be observed in the absence of complement, but lytic activity is completely dependent on the presence of complement. It can be shown that the antibody first unites with its antigen and that this complex then takes up or fixes complement. Complement is a normal protein-complex constituent of the blood, and many assume that it exerts an enzyme-like action on antigens *sensitized* to its action as a result of combination with antibody. Its role in contributing to the defense of a host against an invading parasite is not fully understood, it being able to lyse only a relatively few bacteria (such as the cholera vibrio) after the bacteria have been sensitized by union with antibodies specific for them.

The fact that complement has united with an antigen-antibody complex can be demonstrated by means of the *complement fixation test*. In this test an indicator system is added to the mixture of antigen, antibody, and complement to detect the presence or absence of complement. Since complement reacts with all antigen-antibody complexes, it is therefore possible to use as an indicator system another antigen-antibody complex which undergoes visible change in the presence of complement. Blood cells, after union with antibodies against them, are particularly susceptible to the lytic action of complement, and as their lysis may be observed directly (or indirectly by hemoglobin liberation), they are commonly employed as the indicator system. The complement fixation test rests on the specificity of antigen-antibody reaction and is generally a more delicate or sensitive test than agglutination or precipitation tests alone. Complement fixation was first observed by Bordet in 1901 and is employed to the greatest extent in the detection of syphilitic individuals by means of the Wassermann test. A positive test may be obtained early in the course of syphilis and tends to remain positive as long as the individual is infected.

**The Wassermann Test.** In the Wassermann test blood is collected and allowed to clot, the serum removed, and the complement inactivated by heating the serum at 56° C. Then this serum is mixed with an antigen



obtained from beef heart, which reacts like syphilitic antigen (in the original test a suspension of spirochetes in syphilitic liver was employed). And a small, known amount of complement is added. It is essential to add a small and known amount of complement since all tests must be comparable, also if an excess of complement were present, some complement might remain free in the reaction mixture and cause lysis of the red blood cells subsequently added. The heat-inactivated serum-antigen-complement mixture is incubated for a period of time sufficient to bind

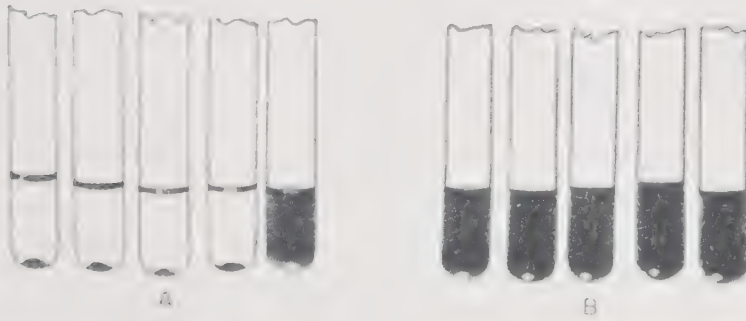


FIG. 21-3 Results of a strongly positive (*A*) and a negative (*B*) Wassermann test. In the first four dilutions of the serum tested in (*A*), no lysis of the red blood cells is observed, and the cells have settled to the bottom of the tubes. Partial lysis is observed in the fifth tube. Complete hemolysis occurred in all dilutions of the serum tested in (*B*); hence serum (*B*) is negative for syphilis.

the complement present in the mixture. The next step in the test is the determination of whether or not complement has been bound; this in turn is dependent on whether or not syphilitic antibody was present in the blood sample. Sheep red blood cells which have been sensitized to the lytic action of complement (when present) by allowing them to react with anti-sheep-red-blood-cell antibody are then added, their only function being to act as an indicator system. The complement in anti-sheep-red-blood-cell serum must be inactivated by heat before it is added to the blood cells; otherwise lysis would be observed. When lysis of the red blood cells occurs, it means that complement was still present, and therefore no syphilitic antibody was present in the individual's serum to react with the syphilitic antigen. When no lysis of the red cells occurs (see Fig. 21-3), the results suggest that syphilitic antibodies were present in the blood of the individual, that they had reacted with the antigen, and that this syphilitic antigen-antibody complex had fixed or bound the complement. Hence, it can be concluded with a considerable degree of accuracy that the individual is syphilitic, provided that he does not



have any infection, such as yaws, which gives rise to a positive Wassermann test. Needless to say, a number of control tests must be run at the same time to determine that all reagents employed in the test are active and functioning properly. The Wassermann test for the detection of syphilitic antibodies (actually a substance called syphilitic reagin which reacts with syphilitic antigen as its antibody should) in the serum of an individual suspected of being syphilitic may be summarized as follows:

### I. PRIMARY REACTION MIXTURE

Syphilitic antigen + Inactivated serum + Small, known amount  $\rightarrow$  No visible reaction  
to be tested of complement

*Were syphilitic antibodies present?* If present, complement would have been fixed.

*Was the complement fixed?* Can be determined by adding indicator system.

### II. INDICATOR SYSTEM

Washed red blood cells + Inactivated anti-RBC serum  $\rightarrow$  RBC's sensitized to the  
action of complement

### III. FINAL REACTION MIXTURE

Mixture I + Indicator II—	{	$\rightarrow$ No hemolysis	Syphilitic
		$\rightarrow$ Hemolysis	Nonsyphilitic

No hemolysis indicates that the serum contained syphilitic antibodies, that an antigen-antibody reaction occurred in I, and that the complex bound the complement.

Hemolysis indicates that the serum did not contain syphilitic antibodies in demonstrable amount; therefore, no antigen-antibody reaction occurred in I, and therefore no complement was fixed in I and hence is free to lyse the sensitized red blood cells of the indicator system II. Individual appears to be nonsyphilitic.

In general, complement fixation tests can be employed for the diagnosis of infectious diseases during the course of which antibodies are formed which give rise to positive agglutination tests or to other antigen-antibody manifestations. Since it is a more sensitive test, it will detect antibodies in lower concentration than can be detected by the other tests. It has been reported to be of particular value in the diagnosis of gonorrhea and other infections in which the agglutination test is of little or no value owing to the low antibody titer generally developed in such infections. It can also be employed in a number of viral infections if suspension of the virus can be obtained.

When a known immune serum is employed in the complement fixation test, the test can be used for the identification of unknown antigens, either free or in a combined state within bacterial or other cells. The test is a very delicate one, and extreme care must be employed in the choice of reagents and their concentrations, in periods and conditions of incubation of the test materials, and in the use of appropriate controls.

The details of the various tests and many of the specific applications have not been considered here and can be found in the standard texts on immunology and serology and in the various clinical laboratory manuals. It should be emphasized that we have considered only the principles of these tests in our discussion and that in actual use numerous factors enter into the proper application of these reactions. Likewise interpretation of the results of these tests requires considerable experience and thought.

**The Schick and Dick Tests.** An *in vivo* application of antigen-antibody reactions (neutralization of toxin) is involved in the Schick test for susceptibility to diphtheria toxin and the Dick test for susceptibility to the toxin of scarlet fever. In these tests, results of a reaction which occurs in local tissues of the test individual are observed. When it is desired to determine whether or not an individual is susceptible to diphtheria toxin, a minute amount of the toxin is injected into the skin. If the individual is susceptible, the toxin will produce a local tissue irritation, and an area of inflammation and swelling results. If the individual is immune, as a result either of having had diphtheria or of having been immunized against diphtheria toxin, no reaction will be observed because the antitoxin in the blood neutralizes the injected toxin. In this type of skin test a positive reaction indicates susceptibility, a negative test meaning immunity, and hence the results of the reaction indicate the reverse of those noted in tests such as the tuberculin test which are based on hypersensitivity to a particular agent. The skin test for susceptibility to diphtheria toxin is known as the *Schick test*, while the test for resistance to the toxin of scarlet fever is called the *Dick test*. Other tests of this nature have been developed for use in man or other animals but in general do not have as wide an application.

**Neutralization Tests.** Neutralization of toxicity tests can be conducted for the detection of exotoxins, using a serum containing a known antitoxin, or for the detection of antitoxin when known toxin is employed. The latter test can be used to determine the efficiency of an immunization procedure. Measured amounts of the serum, or dilutions thereof, are added to constant amounts of the toxin, and the mixtures—after suitable incubation for the reaction to occur—are injected into animals susceptible to the toxin. If the animals fail to develop the symptoms characteristically induced by the toxin alone, and the control animals injected with toxin alone develop characteristic symptoms, the tests indicate the presence of toxin-neutralizing antibodies—antitoxin. The highest dilution of the serum neutralizing the standard toxin serves as a measure of the antitoxin content of the serum.

A similar test is employed with viruses and serves to detect the presence of virus-neutralizing antibodies in serum, or vice versa. This neu-

tralization-of-infectivity test is carried out in a manner similar to that described above for toxins and antitoxins, the virus and the virus-serum mixtures being injected into susceptible animals or into tissue cultures (poliomyelitis), or into developing hens' eggs (mumps), if the virus is capable of multiplying therein. In the latter tests, failure of the virus in the virus-serum mixtures to multiply and produce lesions indicates the presence of neutralizing antibodies and that they have neutralized the virus. Virus by itself is used as a control to show that the virus preparation is an active one.

We have considered the more important serological reactions which can be employed in the laboratory for the identification of either unknown antigens or antibodies, free in solution or combined in bacterial or other cells, provided one of the reactants is known. Certain of these tests will be mentioned again in considerations of the identification of bacterial species or types and of certain infectious diseases.

## CHAPTER 22

### THE ENTEROBACTERIACEAE

Since there are thousands of species of bacteria known to man, it is impossible to consider each species or even each genus in a textbook of general bacteriology. In the long-range plan of nature, the saprophytic forms are of most importance to us, but the pathogenic forms appear to affect us most in our everyday life. The important disease-producing species pathogenic for man are relatively limited in number, probably not greatly exceeding one hundred species. Representative species of both saprophytic and pathogenic species are found in the Enterobacteriaceae, and a general consideration of certain members of this family will illustrate many of the principles of bacteriology, principles which can be applied to other families of bacteria as well.

The family *Enterobacteriaceae* may be defined as a group of nonsporulating, gram-negative rods widely distributed in nature, many of which are parasitic upon plants or animals. Some are motile by means of peritrichous flagella, others nonmotile. The majority of the constituent species grow well on artificial media, and all ferment carbohydrates with the production of acid, or acid and gas. Most members of the family reduce nitrates to nitrites, and many species liquefy gelatin.

The family Enterobacteriaceae is divided (sixth edition of Bergey's Manual) into five tribes, which may be characterized as follows:

- Tribe I. The *Escherichiae*, intestinal parasites and related species fermenting glucose and lactose with the production of acid and gas
- Tribe II. The *Limniae*, species closely related to tribe I, although a few members produce acid only during the fermentation of glucose or lactose. Commonly parasitic on plants
- Tribe III. The *Serratiae*, similar to the above species in biochemical characteristics but characteristically produce a red pigment
- Tribe IV. The *Proteae*, consist of highly pleomorphic rods which ferment glucose and generally sucrose, but not lactose, with the production of acid and gas. Tend to be more proteolytic than other members of this family
- Tribe V. The *Salmonellae*, intestinal pathogens which ferment glucose with the production of acid or acid and gas. A few species ferment lactose with acid but not gas production. Not proteolytic



The Enterobacteriaceae is a fairly homogeneous family, the various species being quite similar in their morphology and general biochemical characteristics, and the division into tribes is made primarily on the basis of their normal habitat and their constituent enzymes as reflected by their fermentative abilities. It is of interest, in connection with our earlier discussion of the evolution of parasitism and pathogenicity, that the evolution of parasitism is well illustrated in this family. The Ser-



FIG. 22-1. Theodor Escherich, discoverer of *Escherichia coli* (*Bacterium coli commune*).

rateae and Proteae consist of saprophytic species capable of utilizing a wide variety of foodstuffs and of producing, under anaerobic conditions, a wide variety of products from carbohydrates and proteinaceous material. They are quite widespread in nature and find conditions suitable for their growth and maintenance in the soil and water, although they may occasionally invade the animal body, generally following establishment of an infection by a pathogenic organism. During the course of time, some of these organisms may have become adapted to growth on living plants and finally developed the ability to invade the tissues of the plant, generally producing only local lesions but occasionally eliciting a more widespread

infection and killing the plant. This would give rise to the Erwiniae, either from the Serrateae, with loss of pigmentation, or from the Proteae. Their dependence on the host does not appear to be as marked as that of the majority of the animal pathogens.

The Eschericheae is a transitional group of organisms in metabolic activity and normal habitat. The genus *Aerobacter* consists of a closely related group of organisms which normally occur in the soil but which may live as commensals (or symbionts) in the intestinal tract of animals. Members of the genus *Escherichia* have become more adapted than the *Aerobacter* species to a commensal or symbiotic mode of life and find conditions in the soil less favorable for their growth and maintenance. In this tribe there are somewhat more specific nutritional requirements than in tribes II, III, and IV, and a gradation exists from *Aerobacter* through *Escherichia* to the still more exacting nutritional requirements of the members of the Salmonelleae.

Members of the Salmonelleae are relatively limited in their nutritional requirements and in their metabolic activities. They have become highly adapted to growth within the animal body and find conditions outside the animal body unfavorable for their existence for any length of time. In this tribe there is progressive loss of metabolic activity and increasing dependence on the host through the Salmonelleae to the Shigellae. However, none of these bacteria have undergone retrograde evolution with loss of synthetic powers to as great an extent as many of the other pathogenic species.

The Erwineae will not be considered here, as they are mentioned in the chapter on infectious disease. The Proteae are of little direct importance to us and will be considered only incidentally to certain of the pathogenic forms. Likewise the Serrateae are of limited importance, although of some historical significance. They are a nuisance at times in that they may cause spoilage of improperly handled milk, giving to milk a red color due to the pigment they produce. Also they will grow on moist, starchy materials such as bread, producing thereon a bloody appearance. This phenomenon was frequently noted in the early days, particularly in the warm, humid Mediterranean countries. When these blood-red spots appeared on the sacramental wafers, they were supposed to be the blood of Christ which appeared as the result of a miracle. The type species, *Serratia marcescens*, is one of the smallest common bacteria and is frequently employed in testing the bacteria-retaining ability of filters employed for the sterilization of heat-labile substances and for the separation of bacteria from suspensions of filtrable viruses. Owing to the rapidity and ease with which it grows and to its characteristic red pigment, it can readily be detected in a filtrate. If it does not pass through the filter, then very likely no other bacteria will pass under the same conditions.

Most informed people know that typhoid fever is caused by a bacterium which may be transmitted by food or water contaminated with the urine or feces of an individual harboring the causative organism. Paratyphoid (typhoid-like) fevers and bacillary dysentery may be transmitted in like manner and are caused by organisms closely related to *Salmonella typhosa*. It might seem offhand that these organisms could be readily detected in food or water, but frequently they are present, particularly in water, in such small numbers that it is very difficult to separate them from accompanying fecal forms such as *Escherichia coli*. Therefore the bacteriologist has been forced to turn detective and to search for clues of the possible presence of these organisms. Pollution of water with fecal material, whether or not contaminated with pathogenic forms, generally contributes two common readily cultivated intestinal bacteria, *E. coli* and intestinal streptococci, to the water in relatively

large numbers. The presence of intestinal streptococci is widely employed in England as an *indicator* of fecal pollution, while the common practice in this country is to look for the presence of *E. coli* and related forms such as *Aerobacter aerogenes*, these and closely related species being collectively known as the coliform bacteria. It should be mentioned that methods have been devised for the isolation of the intestinal pathogens from water known to be polluted with fecal material, but these methods are not suitable for routine use.

### ISOLATION AND IDENTIFICATION OF PATHOGENIC SPECIES

Suspected foodstuffs or fecal material may be streaked out directly on differential media, the principles of which were considered in Chap. 11. Bile salts and dyes are frequently added to the differential media to exert a selective action inhibiting growth of gram-positive species. At the same time the medium must not inhibit the growth of the bacteria which one is trying to isolate. In the separation and identification of members of the Eschericheae and the Salmonelleae, we find beautiful illustrations of the principles of bacterial nutrition, of the classification of bacteria, and of the applications of serological methods. Material suspected of containing Salmonelleae is streaked on lactose agar containing an indicator of fermentation, and after an incubation period the plates are examined for the presence of *colorless* colonies, colored colonies indicating lactose-fermenting organisms. We have seen that the Salmonelleae, with minor exceptions, do not ferment lactose. Colorless colonies, shown to be composed of gram-negative, non-spore-bearing rods, may be fished and inoculated individually in further differential media to aid in the identification of members of the tribe Salmonelleae, which is divided into two genera characterized as follows:

Genus I. *Salmonella*, produces acid and gas from glucose (S. typhosa acid only, differs from *Shigella* in being motile)

Genus II. *Shigella*, produces acid from glucose. Nonmotile

Since members of the genus *Proteus* may also be present at times in fecal matter, this organism must be ruled out and the pathogenic form assigned to its proper genus. Figure 22-2 indicates the biochemical characteristics employed in the differentiation into genera and of genera into the more important pathogenic species.

Biochemical properties such as ability to ferment other sugars, to reduce nitrates, to produce hydrogen sulfide, to liquefy gelatin, etc., serve further to differentiate the species in these genera, and serological tests

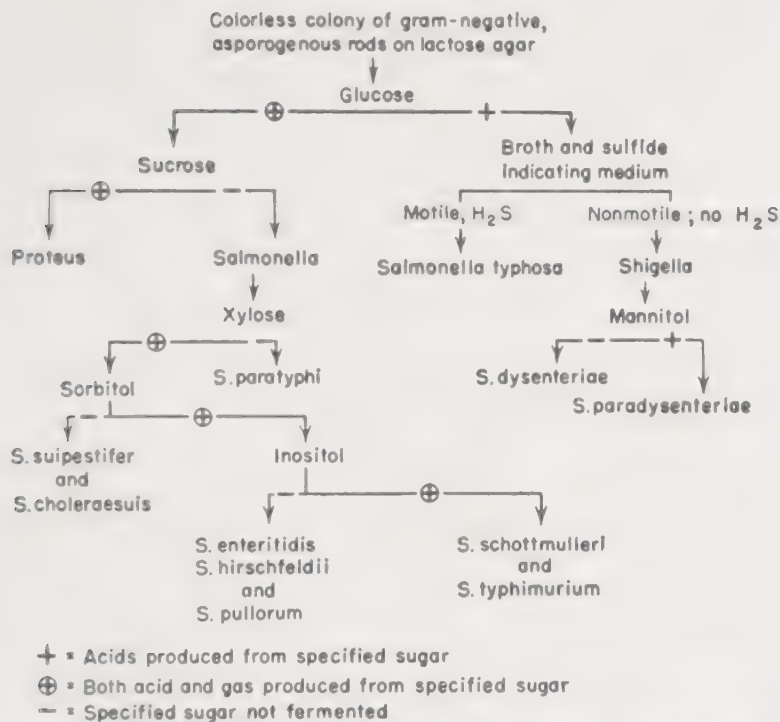




TABLE 22-1 CHARACTERISTIC REACTIONS OBSERVED ON TRIPLE SUGAR IRON AGAR \*

Species	Slant	Butt	H <sub>2</sub> S
<i>Aerobacter aerogenes</i> .....	+	AG	—
<i>Escherichia coli</i> .....	+	AG	—
<i>Escherichia freundii</i> .....	+	AG	+
<i>Proteus vulgaris</i> .....	+	AG	+
<i>Proteus morganii</i> .....	—	AG	+ or —
<i>Salmonella paratyphi</i> .....	—	AG	—
Other <i>Salmonella</i> species...	—	AG	+ or ++
<i>Salmonella typhosa</i> .....	—	+	+
Species of <i>Shigella</i> .....	—	+	—
Species of <i>Alcaligenes</i> .....	—	—	—
Species of <i>Pseudomonas</i> ....	—	—	—

\* + = acid, or H<sub>2</sub>S

AG = acid and gas

— = no acid, or H<sub>2</sub>S

alkaline to yellow in acid solution. However, aerobic conditions prevail on the slant, and in the early stages of growth the sugar for the most part is oxidized to carbon dioxide and water. Most of the glucose will be oxidized by the time heavy growth has been established, and hence there will be no opportunity for glucose to be fermented on the slant, or the extent of aerobic fermentation will be so limited that insufficient acid is produced to develop the acidic color of the indicator. Therefore no color change will be observed on the slant when glucose is the only sugar fermented by the species under examination.

When heavy growth has been established on the slant, oxygen will be consumed by the bacteria near the surface of the growth almost as rapidly as it diffuses into the external layers of cells, and anaerobic conditions will prevail within the bulk of the growth. The outward diffusion of carbon dioxide will also hinder the inward diffusion of oxygen. With the establishment of anaerobic conditions within the heavy growth on the slant (or within colonies on carbohydrate agar in petri dishes), fermentation will become evident along the slant provided that a fermentable sugar is still present in sufficient quantity. This condition prevails if lactose or sucrose is fermented by the organism since these sugars were both initially present in concentrations of 1.0 per cent. Needless to say, that lactose and sucrose will also be fermented in the butt of the tube

by organisms which possess the enzymes to elicit fermentation of one or both of these sugars on the slant. If both the butt and slant assume the acid color of the indicator, all we can tell is that glucose and either lactose or sucrose, or both, have been fermented. But if acid accumulates only in the butt of the tube, then the fermentable sugar is glucose.

Development of the acid color on the slant and in the butt rules out the common enteric pathogens. The inclusion of sucrose in the medium also enables us to rule out *Proteus vulgaris*, which gives colorless colonies on a lactose medium but which is able to ferment sucrose. Also, genera such as *Alcaligenes* and *Pseudomonas*, which might at times be encountered in fecal matter, form colorless colonies on lactose agar, but they are ruled out by the fact that they do not ferment sugars and hence no color change is observed either in the butt or on the slant of the triple-sugar medium.

When an acidie type of fermentation is observed only in the butt of the tube, we know that we are dealing with an organism which ferments glucose but not lactose or sucrose, and therefore, if it is a gram-negative, nonsporeforming rod, it probably is an enteric pathogen. Acid and gas suggests a *Salmonella*, acid alone either *Salmonella typhosa* or a *Shigella*. The inclusion of a soluble iron salt in the medium serves as an indicator of hydrogen sulfide formation, iron being precipitated as the sulfide, which blackens the medium. The inclusion of an indicator of sulfide production considerably increases the differential characteristics of this medium, since most species of *Salmonella*, with the exception of *S. paratyphi*, produce hydrogen sulfide, and *S. typhosa* can be differentiated from the shigellae, as the latter do not produce sulfide. Motility tests may also be carried out with organisms present in water of condensation at the bottom of the slant. A single tube can give much information if advantage is taken of the principles of bacterial physiology! The organisms from the slant can be used to inoculate other carbohydrate media for the determination of additional fermentation characteristics which serve in identifying the species, or they can be used in agglutination tests for serological confirmation and extension of the identification based on biochemical activities. If there is any doubt about the results of tests in these complex media, they should be confirmed in individual sugar media.

In the attempted isolation of *S. typhosa* from water or feces, it is possible to inhibit the growth of coliform bacteria partially by the addition of sodium selenite to broth, which is then inoculated with the sample. Selenite may also inhibit the growth of other bacteria, such as *Pseudomonas*, which may be present in the test sample, and on incubation overnight the microbial population is selectively enriched with respect to *S. typhosa*. Material from this enrichment culture can then be streaked on lactose agar with much greater chances of securing individual colonies of *S. typhosa* without overgrowth of accompanying bacteria.

**Antigenic Structure of Pathogenic Species.** Agglutination tests can be carried out with the organisms isolated and partially identified by means of their biochemical properties. We have considered that many closely related species share one or more antigens in common, and cross agglutination reactions are frequently observed. These cross reactions tend to disappear when the diagnostic sera are highly diluted or have been purified by adsorption of antibodies with antigens shared in common by a number of species. Let us again consider the agglutinin-adsorption technique, which may be illustrated as follows: Suppose organism 1 contains three antigens represented by the letters *A*, *B*, and *C*, and that organism 2 contains antigens *C*, *D*, and *E*. Since both organisms have one antigen in common, an antiserum against one organism will elicit some agglutination when mixed with the second organism. Suppose we wish to have an antiserum specific for organism 1. We could mix the antiserum with organism 2, incubate, and then remove organism 2 from the mixture by centrifugation. Organism 2 would have reacted with antibody against antigen *C*, and this antibody would be carried down with the centrifuged cells; then the supernatant fluid would contain only antibodies against antigens *A* and *B* after the adsorption and removal of antibodies against antigen *C*. Therefore the antiserum so purified would be specific for organism 1, since the antibodies against the antigen shared in common by the two species have been removed. By means of agglutinin-adsorption techniques antisera highly purified in their antibody content and specificity can be prepared, and they are very useful in differentiating between

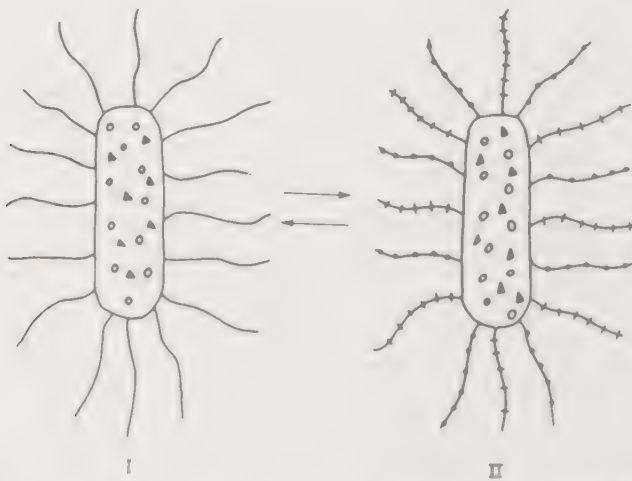


FIG. 22.3 Schematic illustration of diphasic variation in *Salmonella choleraesuis*. I  $\leftrightarrow$  II. Specific flagellar antigen *c* (—●—); Nonspecific flagellar antigens 1 (—○—) and 5 (—●—). Somatic antigens 6 (▲) and 7 (□).

closely related species and in analyzing species for their antigenic structure, i.e., giving an indication of their specific antigenic components.

The use of agglutinin-adsorption techniques and agglutination tests for the antigenic analysis and classification of bacteria is well illustrated with the salmonellae. In these, and in many other species, there is generally more than one antigen in the cell proper. In the Kauffmann-White classification these cellular antigens, usually called the O, or somatic, antigens, are designated by arabic numerals. (Formerly roman numerals were used.) At least thirteen O antigens have been reported in different species of *Salmonella*. The somatic antigens of a particular species tend to be a fairly constant characteristic of the species. Most *Salmonella* species are flagellated, and the flagellar, or H, antigens are of two kinds: those shared by a number of species or types and those characteristic of a particular species or only a few species or types. Organisms showing this variation in flagellar antigens are said to be *diphasic*; i.e., at one time the specific antigens may be present (specific phase), while at another stage of the culture the group, or nonspecific, antigens may be present. A culture may consist entirely of one or the other of the phases or may contain both. Since there is some doubt as to the "specificity" of the H antigens, there is a tendency to refer to the species-specific flagellar antigens as phase 1 antigens and to the less specific, or group, antigens as phase 2 antigens. Phase 1 antigens are designated by small letters, and more than twenty-seven have been described. The group, or phase 2, antigens, designated by arabic numerals, include about six different antigens.

Flagellated (H) organisms agglutinating in the presence of serum containing anti-flagellar antibodies tend to clump together in loose floccules, while a firmer and more dense type of agglutination is observed when

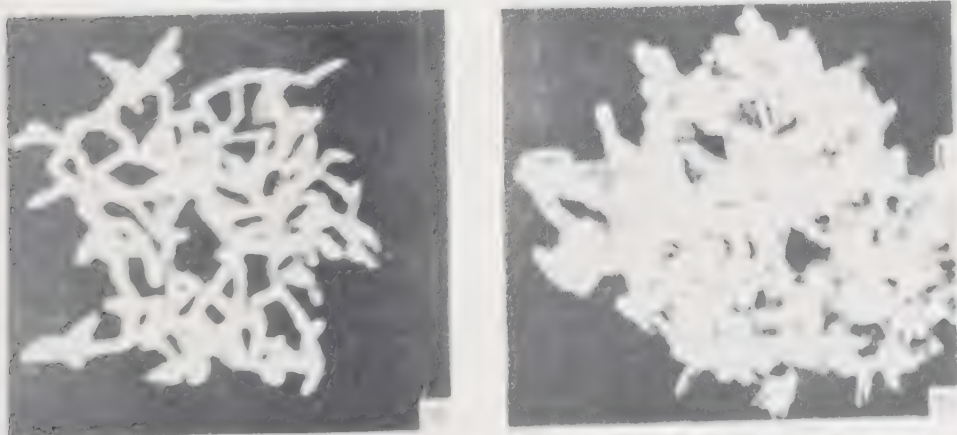


Fig. 22-4. Flagellar (H) and somatic (O) agglutination. [From Peiper, *Journal of Pathology and Bacteriology*, **47**, 1 (1938) and **53**, 431 (1941).]



the reaction is elicited by antibodies against the somatic (O) antigens. Flagellar antigens can be removed from the cells, or at least inactivated, by extraction of the cells with hot ethyl alcohol. Since different procedures are employed in separating the various antigenic constituents of a cell, it must be borne in mind that certain of these antigens may be artifacts, i.e., produced as a result of the chemical treatment, since proteins or complexes with proteins are rather reactive and unstable. However, the existence of numerous antigens in species of *Salmonella* is well established and is of great value in their classification.

The significance of phase variation, which occurs independently of S-R variation, is unknown. (In the S-R variation another change in antigenic structure may be noted, the somatic antigens tending to lose their specificity, which appears to be controlled in the S forms by polysaccharide haptens.) From the results of numerous studies it is possible to assign definite antigenic constitutions, expressed in terms of numbers and letters as just mentioned, to species. For example, the antigenic structure of *Salmonella typhimurium* may be expressed by the formula: (1), 4, (5), 12; i, 1, 2, 3. (The O antigens are indicated first and when the symbol representing an antigen is enclosed in parentheses, it means that the antigen may or may not be present, or is incomplete.) Many salmonellae, differing by only one antigenic component or biochemical property, are given species rank, and final "species" identification is often

TABLE 22-2. ANTIGENIC CONSTITUENTS OF COMMON *Salmonella* SPECIES \*

Group	Species	O antigens	H antigens	
			Specific, or phase 1	Nonspecific, or phase 2
A	<i>Salmonella paratyphi</i>	(1), 2, 12	a	
B	<i>Salmonella schottmuelleri</i>	(1), 4, (5), 12	b	1, 2
	<i>Salmonella typhimurium</i>	(1), 4, (5), 12	i	1, 2, 3
C	<i>Salmonella hirschfeldii</i>	6, 7, (Vi)	c	1, 5
	<i>Salmonella choleraesuis</i>	6, 7	c	1, 5
D	<i>Salmonella enteritidis</i>	(1), 9, 12	g, m	
	<i>Salmonella pullorum</i>	9, 12		
	<i>Salmonella typhosa</i>	9, 12, (Vi)	d	

\* From the studies of Kauffmann, White, and others.

difficult. However, antigenic analysis indicates that there are six main serological groups, A to F, and probably group C may be subdivided into two parts and E into three parts. The grouping of the more common pathogenic species and their antigenic formulas are presented in Table 22-2. Note that *Salmonella typhosa* is closely related in its antigenic structure to group D salmonellae, and for this reason it is now classified as *Salmonella typhosa* rather than as *Eberthella typhosa*. Possibly it evolved from another *Salmonella* by loss of certain nutritional and biochemical characteristics.

**Salmonella Typhosa.** We shall consider *Salmonella typhosa* and typhoid fever, respectively, in some detail, as they represent a well-known pathogenic bacterium and a disease produced in man by the organism under consideration. The infectious nature of typhoid fever was first clearly recognized in 1856 by Budd, who suggested, on the basis of epidemiological evidence, that the disease is transmitted by water contaminated with human feces. The responsible organism was not suggested until 1880, when Eberth noted a gram-negative rod characteristically present in the mesenteric glands and the spleen of persons who had succumbed to the disease. Four years later Gaffky succeeded in isolating the bacterium in pure culture, but some difficulty was encountered in the acceptance of this organism as the causative agent of typhoid fever. The Eberth-Gaffky bacterium was pathogenic in fairly large doses for a number of laboratory animals when injected intravenously or intraperitoneally, but the infection or poisoning did not resemble human typhoid. It was not pathogenic for animals, except possibly chimpanzees, when administered in food or drink, the normal pathway of infection in man. Hence Koch's postulates could not be fulfilled, but serological tests gave support for the belief that the organism isolated by Gaffky was the cause of typhoid. Accidental swallowing of the organisms by laboratory workers finally enabled Koch's third postulate to be fulfilled.

*Salmonella typhosa* is a short, plump, gram-negative rod, its dimensions ranging from about 1 to 3.5  $\mu$  in length and from 0.5 to 0.8  $\mu$  in breadth. It is actively motile by means of peritrichous flagella. *S. typhosa* grows less luxuriantly than *Escherichia coli* on laboratory media and is frequently overgrown by the latter organism. We have already considered that the metabolic activities of the typhoid bacillus are less numerous and less varied than those of the *Escherichia* or other *Salmonella* species. This organism appears to have lost certain synthetic properties possessed by the wild ancestor, as many strains will not grow on synthetic media with potassium salts as the sole source of nitrogen. In particular, these strains appear to have lost the ability to synthesize tryptophan, and this amino acid must be supplied preformed in the medium. However, it is frequently possible to adapt these excreting strains to growth in the ab-

sence of tryptophan by gradually reducing the content of tryptophan in the synthetic medium employed in subculturing the organism. However, the non-tryptophan-requiring variant selected has developed the ability to synthesize this amino acid, since it can be demonstrated in the constituent proteins of the typhoid bacillus. Possibly the tryptophan-requiring form never completely lost this synthetic ability, but the rate of synthesis was so low as to be of little or no value to the organism, training bringing out this latent property. It is of interest in this connection that *S. typhosa* is unable to produce indole from tryptophan, a property possessed by *E. coli* but not by the salmonellae.

In 1934, Felix and Pitt found that different smooth strains of typhoid bacilli differed in their agglutinability by O antiserum, and that the freshly isolated and more virulent strains are the less agglutinable forms. This was shown to be due to the presence of a very labile antigen which Felix designated as the Vi, or virulence, antigen. There is some doubt at present as to the relation between this antigen and virulence, pathogenicity apparently being due in the main to the somatic antigens 9 and 12, which appear to consist of specific carbohydrate-lipoid complexes attached to less specific proteins. These somatic antigens must be present in the bacteria employed in the preparation of antityphoid vaccines if immunization is to be successful.

Dissociation of *S. typhosa* from the ordinary smooth form to the rough form is frequently noted, with or without loss of flagella, and hence four main types may be observed: smooth motile, smooth nonmotile, rough motile, and rough nonmotile. The *S-R* variation appears to be accompanied by a change in serological specificity involving the somatic, or O, antigens; the Vi antigen is apparently independent of this variation and may be present with or without O antigen. Hence, a series of antigenic variations is possible, the organisms possessing H, O, and Vi antigens separately or in combination. The *S-R* transformation may take place in the body as well as in the test tube, healthy carriers of *S. typhosa* frequently carrying readily agglutinable, avirulent rough strains. Variation may also be observed in biochemical characteristics, some strains fermenting xylose and others not, a difference to which some workers assign epidemiological significance. Some variants of the typhoid bacterium have been isolated which possess the full complement of antigens characteristic of the virulent form. These variants, however, require a purine for growth, this purine was not required by the virulent parent strain, and apparently was not supplied by the tissues of the test animal. Nutritional factors as well as antigenic structure appear to play a role in determining the virulence of an organism.

The typhoid bacillus has become adapted to a parasitic mode of life in man, and when found outside the human body, it can generally be treated



more or less directly to the discharges of a typhoid patient or of a carrier of the organism. Laboratory studies have shown that the bacillus may survive for three months or more in sterile water but that it disappears much more rapidly in unsterilized water. Under ordinary conditions it does not multiply in water, even in the presence of considerable organic matter, and the danger of infection from sewage-polluted water is greatest when the sewage is fresh. The persistence of typhoid bacilli in soil and in fecal matter is much longer than in natural waters, viable organisms having been found in contaminated soil as long as two months after contamination has occurred.

**Typhoid Fever.** After an incubation period of about a week, the disease becomes evident in man, the clinical symptoms being variable and not too well defined. The common symptoms are headache, lack of appetite, nosebleed, the development of rose-red spots on the abdomen, muscular weakness, diarrhea, and a gradually increasing fever during the first 10 days. The fever lasts for 3 to 4 weeks, and recovery is gradual. The disease varies from mild, frequently unrecognized cases known as "walking typhoid" to severe cases, the mortality rate being about 10 per cent. On autopsy the intestinal walls are generally observed to be extensively ulcerated, "Peyer's patches" being particularly involved and containing large numbers of the bacilli. The intestinal wall may perforate with a resulting peritonitis, often involving other bacteria as secondary invaders. Secondary mixed infections elsewhere in the body, especially involving the pyogenic cocci and pneumococci, are not uncommon.

In addition to the more or less constant symptoms associated with typhoid fever, there are certain other pathological conditions which may be produced. Inflammation of the urinary bladder (cystitis) sometimes occurs, and the gall bladder is frequently heavily infected and severely inflamed. The spleen and liver may contain large numbers of bacteria, generally in masses. Suppurative and inflammatory processes may also be produced in many parts of the body; the osseous system in particular is open to invasion, and *S. typhosa* can frequently be isolated from the bone marrow. Other organs of the body are rarely invaded by this organism but under certain conditions almost any organ may be attacked. It is readily apparent that typhoid fever is the result of a generalized infection rather than a localized one.

The intestine has long been regarded as the main portal of entry of *S. typhosa* into the body, the bacteria generally entering the intestinal tract as contaminants in water, milk, or food. They pass through the stomach, enter the upper intestines, and establish an infection in the intestinal walls, particularly in the lymphoid tissue, with consequent inflammation. From this original site of infection the bacteria may spread



into the general lymphatic system and the spleen. The bacteria can spill over into the blood stream in large numbers from the lymph nodes with the establishment of a bacteremia early in the course of the disease. However, there is some doubt as to whether or not the organisms multiply in the blood stream. Although there are no antibodies in the blood stream early in the course of the disease, yet the bacteria soon disappear from it for a time as a result of the phagocytic activity of the reticulo-endothelial system. The bacteria continue to proliferate in various tissues, e.g., in about 80 per cent of recognized cases specific eruptions (rose spots) which contain large numbers of bacilli may be observed on the abdomen, and from the tissues they later invade the blood stream again. By this time antibodies have generally been formed in appreciable amounts, and they may, with the aid of complement, induce lysis of the bacterial cells. Bacteriolysis may be accompanied by the liberation of endotoxic substances, particularly the Vi and O antigens, with the establishment of a septicemia. By the end of the first week or ten days, millions of bacteria escape from the Peyer's patches, enter the intestinal contents, and are excreted in the feces. Little or no multiplication takes place in the intestinal matter. Typhoid bacilli are also found in the urine, frequently many millions per milliliter, in about 25 per cent of the cases of typhoid fever.

The laboratory tests are culturing the blood, urine, and feces for the presence of *S. typhosa* and agglutination tests with the patient's serum against known cultures of this pathogen. The laboratory procedures and findings depend on the stage of the disease as suggested by the above discussion. Blood cultures may be positive during the first invasion of the blood stream in the early days of the infection and may become positive again if the blood stream is invaded for the second time. Stool and urine cultures generally become positive after a week or ten days, and antibodies appear in the blood stream at about the same time, hence agglutination tests may be carried out at this time, testing for the presence in the patient's blood of antibodies against *S. typhosa*. It must be borne in mind that the individual may have been vaccinated against typhoid, and in this case a marked increase in the concentration of antibodies at this stage of the infection is of diagnostic significance. Detection of *S. typhosa* in fecal matter is not always an easy procedure, and full advantage must frequently be taken of the various selective and differential media available.

Stool cultures may be positive for long periods after recovery from typhoid fever. About 5 per cent of individuals recovered from the infection remain carriers for several months and a few for life. In the chronic carrier state the focus of infection is usually the gall bladder in the case of the fecal carriers (the most common) and the bladder in the

case of the urinary carriers. A higher percentage of women than men remain carriers, and there appears to be a greater tendency for the carrier state to develop in older individuals. Isolation of typhoid bacilli from carriers is frequently difficult, and several attempts may be necessary as the organisms may be eliminated intermittently. Antibody to the Vi antigen tends to remain in fairly high titer in carriers and to disappear from noncarriers; hence Vi-agglutination tests may be of value in recognizing the carrier state. Removal of the gall bladder has been effective in eliminating the carrier state from about 70 per cent of surgically treated cases.

**Control of Typhoid Fever.** The control of typhoid fever is probably one of the greatest achievements in the application of the principles of bacteriology to our everyday life. Typhoid was for a long time one of the most common of the serious infectious diseases and one of the leading causes of death. Today it is rare in civilized countries, generally occurring only in endemic form or in small localized epidemics frequently traceable to a carrier. Recognition of the pathways of transmission of the infectious agent and the application of effective methods for the prevention of this transmission are responsible for the reduction in our large cities of from 20.5 cases per 100,000 inhabitants in 1910 (when some sanitary precautions were already in effect) to 0.26 in 1937. Sanitation has accounted for this to a great extent although vaccination of individuals, particularly soldiers, working under unsanitary conditions has been of some value in eradicating this former plague of mankind.

Typhoid fever appears to be contracted only by the ingestion of typhoid bacilli, there being some evidence that the organisms invade the body by way of the tonsils or gastric mucosa at times as well as through the intestinal mucosa. The disease may be transmitted by any means which allows the transfer of typhoid-contaminated feces or urine to the mouth, although such transfer does not necessarily mean that an infection will be established. Of the various means of transmission the four F's—Feces, Fingers, Flies, and Food—are the most important. Fingers or flies become contaminated with *S. typhosa* present in excreta and transfer the bacteria to food, and in food they gain entrance to the body. Excreta from the patient must be disposed of in such a manner that the organism is not spread to others and carriers must be prevented from handling food that will be consumed by susceptible individuals. "Typhoid Mary" was the most famous carrier in the past half century. In 1901 she developed typhoid fever and, as was discovered later, became a chronic carrier the month after Typhoid Mary accepted a job as cook in a private home, the landlady in the same home developed the disease. In the next year Mary obtained a job as cook in a different home, and soon seven members of the family were ill. During the next four years new jobs followed, and

about 20 cases of typhoid were associated with her activities. She was held by the New York Department of Health for several years, but in 1914 became a cook under an assumed name in a hospital, and an outbreak of 25 cases followed. She was later taken into custody again and held for many years, not without considerable legal difficulty. Total known cases directly attributable to her were 51 in 10 outbreaks, and no doubt many more unknown cases. She may also have been the source of the original contamination responsible for an outbreak of over 13,000 cases in 1903 in Ithaca, N.Y. She died on November 11, 1938, of natural causes. This case and many others show that our foods may easily become contaminated with excrement and that food handlers must be carefully watched, particularly if they have been known to have had typhoid fever. Flies, as well as fingers, may carry the pathogen from excreta to food. Need more be said? Shellfish from sewage-contaminated beds have also been responsible for epidemics of typhoid.

Extensive outbreaks of typhoid fever have generally been traced to contaminated water or milk, the supply of which was used by large numbers of people. Milk-borne typhoid was second to water-borne epidemics up to the general introduction of pasteurization, since which time milk has become unimportant as a vehicle of transmission except where pasteurization is not employed. The infectious agent in the milk did not come from the cattle but from contaminated handlers of the milk. Water-borne outbreaks can be traced to the pollution of a water supply with sewage containing excreta from typhoid patients or carriers. It can be prevented by efficient purification of a water supply. A typical example was observed in a Midwestern town during the depression years. The water supply was from a river near the town, and an economy-minded municipal administration decided to stop chlorination of the town's supply. Within a short time an epidemic of 282 cases with 29 fatalities occurred in a population of approximately 1,500 people. The outbreak was traced to pollution of the river water with excreta from carriers living several miles up the river. Epidemics have also been traced to the use of contaminated water for washing milking equipment. Unwise economy in sanitation does not pay dividends.

Immunity to typhoid fever appears to be of a high order, one attack conferring permanent but not necessarily absolute immunity. Immunization with heat-killed organisms, providing they are in the antigenic phase characteristic of the fully virulent organisms, confers a marked increase in resistance upon the immunized individual. Frequently the paratyphoid bacteria are also incorporated in the vaccine for the purpose of immunizing at the same time against not only typhoid fever but against paratyphoid fevers, which may be transmitted in the same manner as typhoid and which likewise affect the gastrointestinal tract.



**Paratyphoid Fever.** Two species of *Salmonella*, *S. paratyphi* (paratyphoid A) and *S. schottmuelleri* (paratyphoid B), generally give rise to a typhoid-like disease in man. Many cases of paratyphoid fever show a tendency to run a mild course and are marked by sudden onset with chills but otherwise are similar to typhoid fever cases. The only method for accurately differentiating between these infections is isolation and identification of the causative organism. Like typhoid, paratyphoid fevers are transmitted by contaminated food or drink, and their spread may be prevented by the same means employed for the control of typhoid fever. The incidence of paratyphoid fevers is in general much lower than that of typhoid, and the fatality rate is very low.

Several species—*S. enteritidis*, *S. choleraesuis*, and *S. typhimurium*—may cause acute gastroenteritis in man. This type of infection is quite different from typhoid and paratyphoid fevers in that the incubation period is very short, often a few hours, the onset is sudden, and the symptoms are those of a severe digestive upset rather than of a true infectious disease. Diarrhea, vomiting, and abdominal cramps are the usual symptoms; fever may be slight, and generally recovery is rapid with few fatalities. The acute symptoms are generally the result of eating food which has been heavily contaminated with the particular species of *Salmonella* involved. Infection of the food is at times by human carriers but more generally by excreta from rats or mice which are infected or are carriers. Meat from infected animals may also serve as a source of infection. Proper cooking will kill these pathogenic forms, but if they are not killed, or subsequently gain entrance into cooked foods which are allowed to stand for a few hours in a warm place (70° F.), they will multiply to a considerable extent. The symptoms produced are those of an intoxication rather than of an infection, although the organisms do multiply in the intestinal tract and establish infection therein.

**Dysentery.** Clinical dysentery can be caused by several species of *Shigella* or of *Salmonella*, and by colon-like bacteria, a filtrable virus, or by the amoeba, *Entamoeba histolytica*. Organisms of the *Shigella* group are the most common cause of bacillary dysentery, the infection being confined to the intestinal tract and being characterized by the presence of blood and mucus in the stools. The principal species are *Shigella dysenteriae* and *S. paradyserteriae*; *S. sonnei*, *S. ambigua*, *S. alkalescens*, and *S. flexneri* at times being responsible agents. The latter two bacteria are closely related to *E. coli* and may belong in an intermediate group. These bacteria can be differentiated from the salmonellae by their failure to produce gas on carbohydrate media, their failure to produce hydrogen sulfide, their lack of motility, and by specific agglutination. *Shigella* is a less antigenically complex genus than is the genus *Salmonella*, usually containing one or at the most two major somatic antigens. *Shigella*



*dysenteriae* produces a potent exotoxin (Shiga toxin), the other pathogenic species of this genus producing only endotoxins which are released on autolysis of the cells. The Shiga toxin is a lipod-carbohydrate-protein complex, the antigenicity being controlled by the polysaccharide and toxicity being due to the protein fraction.

Dysentery is more difficult to prevent than the typhoid-paratyphoid fevers, being transferred primarily by carriers who infect the food, milk or water before consumption. Pasteurization and chlorination do reduce the incidence of the disease, but scrupulous cleanliness in the handling of food by all workers is required for a more complete control.

## CHAPTER 23

### MICROBIOLOGY OF INFECTIOUS DISEASE

It is impossible to consider all organisms pathogenic for plants and animals in a text of this size. The pathogens are widely scattered amongst the viruses, the bacteria, the higher fungi, the protozoa, and even somewhat higher forms of life such as the small worms. These organisms could be mentioned individually, each in the little niche it occupies in the common classifications of the various forms of life. Such a consideration is of little practical importance to the average student with general interest in the life about him, even if it were limited to the agents infectious for man. Infectious diseases are similar in all instances in that they represent a struggle between the cells of the pathogenic species and of its unwilling host. No attempt will be made, therefore, to cover all pathogens and the infections which they elicit, and the discussion to follow will be primarily limited to the more common species pathogenic for man. These species can be roughly classified on the basis of staining characteristics and morphology as shown in the accompanying key.

#### SIMPLIFIED KEY TO BACTERIA PATHOGENIC TO MAN

##### *Gram-positive Cocci*

##### *Micrococcus:*

Clusters of cocci, characteristically pigmented  
*pyogenes* var. *aureus* (*Staphylococcus aureus*) Carbuncles, furunculosis, pyemia

Characteristically in groups of four (tetrads)  
*tetragenus* (*Gaffkya tetragena*) ..... Secondary infections

*Streptococcus*, cocci in pairs or chains, characteristic hemolysis on blood agar

Complete hemolysis  
*pyogenes* ..... Infections of teeth, tonsils, sinuses, and wounds; erysipelas; puerperal sepsis; bronchopneumonia; septic sore throat; scarlet fever; rheumatic fever

Partial hemolysis, greenish discoloration  
*salvarius* ..... Endocarditis

*Diplococcus*, encapsulated diplococci, soluble in bile  
*pneumoniae* ..... Pneumonia (pneumococcal)

SIMPLIFIED KEY TO BACTERIA PATHOGENIC TO MAN (*Continued*)*Gram-negative Cocci*

<i>Neisseria</i> , diplococci	
<i>gonorrhoeae</i> .....	Gonorrhea, ophthalmia neonatorum
<i>meningitidis</i> .....	Epidemic meningitis

*Gram-negative Rods*

<i>Pseudomonas</i> , polarly flagellated, usually pigmented rods	
<i>aeruginosa</i> .....	Secondary invaders, infections of urinary tract
<i>Proteus</i> , motile, pleomorphic rods. Spreading colonies on agar .....	Secondary invaders, urinary-tract infections
<i>Salmonella</i> , characteristically found in the intestinal tract, easily cultivated, fermentative.	
Motile or nonmotile	
<i>typhosa</i> .....	Typhoid fever
<i>paratyphi</i> , <i>schottmuelleri</i> .....	Paratyphoid fever
<i>typhimurium</i> , <i>enteritidis</i> , <i>pullorum</i> , and <i>choleraesuis</i> .....	Food poisoning
<i>Shigella</i> , similar to <i>Salmonella</i>	
<i>dysenteriae</i> and <i>paradysenteriae</i> .....	Bacillary dysentery
<i>Brucella</i> , small rods, inactive on carbohydrates	
<i>abortus</i> , <i>melitensis</i> , and <i>suis</i> .....	Undulant fever, contagious abortion in cattle
<i>Malleomyces</i> , small rods, tendency to form filamentous, branching cells, usually associated with horses	
<i>mallei</i> .....	Glanders, farcy
<i>Pasteurella</i> , small, bipolar-staining rods, usually associated with animals and spread by insects	
<i>pestis</i> .....	Plague (bubonic), pneumonic plague
<i>tularensis</i> .....	Tularemia
<i>Hemophilus</i> , small, at times filamentous, rods which may require blood for best growth	
<i>pertussis</i> .....	Whooping cough
<i>influenzae</i> .....	Bronchopneumonia
<i>ducreyi</i> .....	Chancroid

*Gram-positive Rods*

<i>Bacillus</i> , aerobic, sporeforming rods	
<i>anthracis</i> .....	Anthrax
<i>Clostridium</i> , anaerobic, sporeforming rods	
<i>tetani</i> .....	Tetanus
<i>perfringens</i> , <i>novyi</i> , <i>septicum</i> , <i>histolyticum</i>	Gas gangrene
<i>botulinum</i> and <i>parabotulinum</i>	Food poisoning

SIMPLIFIED KEY TO BACTERIA PATHOGENIC TO MAN (*Continued*)*Gram-positive Rods (Continued)*

- Corynebacterium*, irregular, club-shaped rods.  
 Metachromatic granules and barring usually  
 observed  
*diphtheriae* ..... Diphtheria
- Mycobacterium*, acid-fast rods  
*tuberculosis* (human and bovine strains) ..... Tuberculosis  
*leprae* ..... Leprosy
- Actinomyces*, branching, filamentous forms, break-  
 ing into rods  
*bovis, madurae, and graminis* ..... Actinomycosis, Madura foot

*Gram-negative Spirilla*

- Vibrio*, short, comma-shaped rods, polarly flagel-  
 lated  
*comma* ..... Asiatic cholera

*Gram-positive Spirilla*

- Spirillum*, rigid, spiral, lophotrichously flagellated  
 rods  
*minus* ..... Ratbite fever

*Spirochetes*

- Treponema*, slender spirals, difficult to stain  
*pallidum* ..... Syphilis
- Borrelia*, coarse, flexible spirals  
*recurrentis* ..... Relapsing fever
- Leptospira*, finely coiled organisms with hooked  
 end or ends  
*icterohemorrhagiae* ..... Jaundice and meningitis

There are four main periods in the course of an infectious disease, as was illustrated in the discussion of typhoid fever. These can be summarized as (1) contamination of the host and entry of the parasite into susceptible tissue; (2) a period of incubation during which the parasite multiplies, this period ending with the appearance of definite symptoms of the disease; (3) the period of obvious illness; and (4) the period of recovery if the host survives. It is quite likely that the fate of the host is dependent to a great extent upon the changes that are initiated during the second period, provided that no treatment is given which would interfere with the normal course of events.

The use of vaccines, where effective, has served to reduce the incidence of certain infectious diseases by preventing establishment of the parasite, while the chemotherapeutic agents are employed during the second and third stages and reduce both the severity and the fatality rate. Con-



trol of the disease at an early stage also reduces the chances for the parasite to pass from the infected host to another susceptible one. Chemotherapy, however, is not 100 per cent effective, and neither are many of our control measures. This statement is illustrated in Table 23-1, in which numbers of cases and of deaths from the more common reportable diseases in California are summarized.

TABLE 23-1. CASES AND DEATHS FROM REPORTABLE DISEASES, CALIFORNIA, 1954 \*

Reportable disease	Cases	Deaths
Anthrax.....	—	—
Botulism.....	6	3
Brucellosis (undulant fever).....	48	1
Chickenpox (varicella).....	47,084	7
Conjunctivitis, acute infectious, of the newborn....	9	—
Diphtheria.....	39	4
Encephalitis, infectious.....	656	53
Food poisoning.....	1,461	—
German measles (rubella).....	7,021	—
Gonococcus infection.....	16,012	1
Hepatitis, infectious.....	2,233	70
Influenza, epidemic.....	377	53
Leprosy (Hansen's disease).....	13	—
Lymphogranuloma venereum.....	53	—
Malaria.....	41	1
Measles.....	60,029	10
Meningitis, meningococcal or meningococcemia....	309	69
Mumps.....	32,885	3
Pertussis (whooping cough).....	4,985	16
Pneumonia, infectious.....	2,268	976
Poliomyelitis, acute anterior.....	4,496	113
Psittacosis.....	64	1
Rabies, human.....	1	1
Rheumatic fever, acute (under 21 years of age)....	232	12
Salmonella infections (exclusive of typhoid fever)...	997	7
Shigella infections (bacillary dysentery).....	1,088	12
Smallpox (variola).....	—	—
Streptococcal infections, respiratory, including scarlet fever.....	8,391	8
Syphilis.....	6,845	369
Tetanus.....	47	23
Tuberculosis (all forms).....	7,904	1,219
Tularemia.....	12	—
Typhoid fever.....	109	4

\* Source: State of California, Department of Public Health, Death and Mortality Records.

In many instances control of infectious diseases is accomplished most readily by breaking the chain through which the parasite is transmitted. The infectious agents tend to leave the body by definite routes and to be spread by vehicles such as air, water, food, insects, and so on. Similarly, the agents tend to enter the host through definite portals, the most frequent being the respiratory tract (mouth and nose), the gastrointestinal tract, and breaks in the superficial mucous membranes and skin. A limited number apparently can pass through intact external barriers, while others commonly gain entrance to the deeper tissues as the result of the bite of an infected or contaminated insect. Major advances in the control of the spread of infectious agents have been made in most instances by breaking the chain of spread of particular species through various means such as (1) reducing the susceptible population by immunization procedures; (2) pasteurization or other treatment of food and drink; (3) efficient disposal of wastes and sewage; (4) control of insect or other vectors; (5) quarantine or avoidance of crowds or infected persons; and (6) in the case of plant diseases, selection of races resistant to the infectious agent.

From the public-health viewpoint a classification of the pathogens on the basis of the methods by which they are transmitted and of the portal of entry into the body, together with an understanding of the organism, is of value in combating the spread of infectious agents. The organisms pathogenic for man, regardless of their position in the scale of life, can be grouped under four broad headings: those most commonly transmitted in food or drink, those transmitted in respiratory secretions, those transmitted by intimate contact with contaminated material or infected individuals, or those transmitted by insects. In the ultimate analysis the portal and mode of entry of the infectious agent into the host are intimately associated with biological characteristics of the host and of the parasite, and classification along these lines will be employed as a guide in the discussion to follow.

#### INTOXICATIONS AND INFECTIONS TRANSMITTED BY FOOD AND DRINK

In the preceding chapter we considered the more common pathogenic species of the *Enterobacteriaceae*, noting that the organisms of the typhoid group generally entered the body in contaminated food, water, or milk. After reaching the intestinal tract, they frequently invade the tissues and may spread throughout the body with the development of a generalized infection. Some of the organisms escape from the body in urine or feces and can be carried from these sources into the food or drink of other persons. Other species of *Salmonella* rarely produce a general-

ized infection in man but do produce a poisoning as a result of the ingestion of large numbers of bacteria, or of their products, in food or drink. These bacteria may continue to multiply for a time in the intestinal tract. Sufficient irritating or toxic material is liberated to produce clinical symptoms generally within 6 to 12 hr. after ingestion of the contaminated material. The evolution of the infection is rather slow and is characterized by gastrointestinal upsets, high fever, and general malaise. The responsible organism frequently can be isolated from the feces. Recovery from the poisoning is usually complete within a few days, the fatality rate being very low.

The principal species of *Salmonella* concerned in salmonella food poisoning (infection) is *S. typhimurium*. Other species of *Salmonella*, however, may cause this type of disease. In most outbreaks of this type of food-borne infection, the food concerned is meat, fish, or milk, frequently in a form such as meat pie, sausages, or puddings which require considerable handling in their preparation. Healthy carriers can contaminate the food during the course of its preparation; cooking may not be of sufficient duration or at a high enough temperature to kill all the bacteria present (particularly in the center of a mass of food such as a meat pie), and these organisms can grow rapidly in such food if it is not refrigerated. In other instances the food may be contaminated by rats or mice which are infected with *Salmonella* species, or the organisms are at times present in meat obtained from infected animals. Thorough cooking and immediate consumption of the cooked food (or proper refrigeration) serves as the best method for the control of food-borne infections.

**Food Poisoning.** The food-borne infections just mentioned are frequently spoken of as food poisonings but are caused by bacteria, or their autolytic products, transmitted in food, infection or poisoning being facilitated by growth of the organisms in the food before it was ingested. There are two principal kinds of true food poisoning, a disease in which the symptoms are produced by poisonous principles, exotoxins, liberated in the food as a result of the activities of the bacteria concerned. These are staphylococcus food poisoning and botulism, and they can be produced in the body by the ingestion of exotoxin free from bacteria.

Staphylococci (*Micrococcus pyogenes*) are always found on the human skin, and it is not surprising that they give rise to the most frequent type of true food poisoning. The variety *aureus* is the most common producer of the enterotoxin of staphylococcus food poisoning, but the actual toxin-producing staphylococci do not fall into any clearly defined group on the basis of cultural or biochemical characteristics. All that can be said with certainty is that some do, others do not, produce the exotoxin to which the human intestine is so sensitive. The ability to produce this enterotoxin can be determined by cultivating the strain of staphylococcus under

question in semisolid agar in the presence of 20 to 25 per cent carbon dioxide and testing sterile filtrates for the presence of the toxin by feeding tests in monkeys or in human volunteers.

Creamy, starchy foods, such as éclairs, cream puffs, or cake fillings, salads or sandwiches containing mayonnaise, ham, and tongue are the usual source of staphylococcus food poisoning, although other foods can also be the source of the poisoning. The food is easily contaminated with staphylococci, which will develop in it if the food is allowed to stand for 6 to 10 hr. in a warm place. During this time the staphylococci can produce sufficient toxin to elicit clinical symptoms in man following consumption of the food. The incubation period is short, generally 2 to 6 hr., and the onset of the symptoms is rapid and violent, with nausea, vomiting, diarrhea, and sometimes collapse. Recovery is generally quite rapid, being complete in 24 to 48 hr. Streptococci have been incriminated in some outbreaks of food poisoning, but the general opinion appears to be that they cause a mild enteric infection rather than a poisoning by a preformed enterotoxin.



FIG. 23-1. Gram stain of *Clostridium botulinum*.

**Botulism.** This is a rather infrequent but extremely fatal type of food poisoning caused by an exotoxin formed during the prior growth of the anaerobe, *Clostridium botulinum*, in food. *C. botulinum* is a gram-positive, peritrichously flagellated rod, 0.5 to 0.8 by 3.0 to 8.0  $\mu$ , with rounded ends and central to terminal oval spores. The organism is a strict anaerobe, ferments a number of sugars with the production of acid and gas, utilizes amino acids primarily by means of coupled oxidation-reduction reactions between pairs of amino acids, and has slight proteolytic power. Studies of Meyer and others indicate that there are five main types, designated A, B, C, D, and E, on the basis of the toxin formed, the toxin produced by one type not being neutralized by antitoxins against the other types. In the 1948 edition of Bergey's Manual, two distinct species are recognized, *C. botulinum* and *C. parabotulinum*, the latter being highly proteolytic. Correct classification of this group of organisms awaits further work, but they are all characterized by the production of closely related, extremely powerful exotoxins, which attack primarily nervous tissue after either ingestion or injection.

*C. botulinum* is a saprophyte widely distributed in the soil. When it gains entrance to neutral or slightly alkaline cooked foods, it can develop



if anaerobic conditions prevail. The spores in many instances are somewhat heat-resistant, but are destroyed within 4 to 5 min. by steam under pressure at a temperature of 120 C. Botulism is not spread by fresh foods, all human cases having originated from foods which were improperly processed, heated either for too short a time or at too low a temperature to destroy the spores. The spores germinated on standing under the anaerobic conditions in the processed material, and the exotoxin was produced during the growth of the organism. Many cases of botulism have developed from improperly processed sausages and from canned meats or vegetables such as string beans or corn. Commercial canning procedures at the present time are based upon the time required to kill the spores of this organism. Home-canned, nonacidic foods should always be thoroughly boiled for at least 10 min. before they are consumed if there is the faintest indication that spoilage may have occurred, the toxin itself being destroyed by boiling.

The symptoms of botulism frequently develop within 24 hr. of the ingestion of food containing preformed botulinus toxin. This toxin is a protein of high molecular weight, approximately 900,000. It passes unchanged through the walls of the digestive tract, differing in this respect from the toxins of diphtheria and of tetanus, which are not toxic by way of the mouth, and reacts with nervous tissue. There is no marked intestinal upset as observed with other types of food poisoning, and the first symptom is generally a disturbance of vision followed by paralysis of the eye muscles, of the throat muscles, and general muscular weakness. Death is caused in most instances as a result of paralysis of the respiratory center. Although specific antitoxins are available, the disease runs such a rapid course and is so well established before it is diagnosed that antisera are of little therapeutic value. Effective control consists in the proper processing of foods, the details for which can generally be obtained from state or county health officials or agricultural extension services, and in refusing to taste any foodstuff which appears to have undergone the slightest amount of decomposition or change. Several cases of, and death from, botulism have been traced to tasting no more than a drop of the suspected food.

Botulism is an intoxication not only of man but also of animals. Chickens are frequently fed spoiled foods, and a number of outbreaks of botulism in flocks have been traced to this practice. The first symptom in chickens is generally a loss of control of neck muscles, hence the name applied to this poisoning—limber-neck. Spoiled grass, fodder, and silage have been responsible for outbreaks in horses and cattle, and the disease has also been recognized in flocks of wild ducks.

**Asiatic Cholera.** Cholera is a severe infection (not a poisoning) of the intestinal tract and is produced by *Vibrio comma*, the cholera vibrio.

This organism is transmitted in fecal material in a manner similar to the other intestinal pathogens. It is a short, comma-shaped, polarly flagellated, gram-negative organism which, while it ferments a number of sugars with the production of acid but no gas, does not grow readily under anaerobic conditions. It is particularly characterized by the ability to grow in media as alkaline as pH 9.2, an alkalinity inimical to the growth of other intestinal parasites. No exotoxin, with the exception of the hemolysin produced by the El Tor strain, is produced, and the symptoms in man are the result of rapid growth of the bacteria in the small intestine and the liberation of intracellular constituents with toxic properties. A toxic polysaccharide-lipide complex identified with the somatic O antigen, a toxic protein fraction, and a fever-producing fraction have been isolated from the organism.

Mild cases of cholera cannot be distinguished readily from mild attacks of food poisoning by staphylococci or salmonella food infections, diagnosis being made on the finding of cholera vibrios in the stools. Frequently the symptoms are more pronounced, the endotoxins destroying large areas of the intestinal walls, causing them to be shed in small flakes. As a result of this breakdown of tissue, considerable quantities of body fluid flow into the intestinal tract. This combined action gives rise to the *rice-water* stools characteristic of the disease. The organism shows little or no tendency to invade the body proper. It can be readily identified in the stools because of the large numbers of vibrios present and the previously mentioned fact that it can be cultivated on a highly alkaline medium inhibitory to most fecal species.

The control measures are the same as for typhoid and paratyphoid fevers, purification of water supplies being the most important single measure. Immunization with properly prepared vaccines appears to be of considerable value for the protection of individuals in areas where cholera is endemic or is present in epidemic form. Human or animal carriers do not appear to be involved in the spread of this disease, the vibrios apparently being maintained in individuals with unrecognized (subclinical) cases of the disease.

**Brucellosis.** Brucellosis is primarily a disease of goats, cattle, and swine which can be transmitted to man by direct contact with infected animals or by consumption of their milk or milk products. Infection in animals generally produces few symptoms other than abortion, but the animals may harbor the organisms over a long period of time and secrete them in their milk. Man is quite susceptible to infection with *Brucella*, but the majority of infections appear to be so mild as to pass unrecognized. Agglutination and specific allergic (brucellergen) tests, however, are positive in many adults. The causative organisms pass through the intestinal mucosa and invade the tissues, local or focal lesions

being produced in various parts of the body. The infection in many instances is characterized by an undulating type of fever in man, hence the common name undulant fever for the disease. It is also known as Malta fever since the disease is prevalent in goats and in man on the island of Malta, and the causative agent, *Brucella melitensis*, was first isolated there by Bruce. Two other species have been isolated from cattle and from swine. It has been suggested that these species, *B. melitensis*, *B. abortus*, and *B. suis*, originated from one original species on adaptation to cattle, goats, and swine. The brucellae are small, gram-negative, asporogenous, nonmotile coccobacilli of the family Parvobacteriaceae. They are exacting in their growth requirements, growing most readily on liver-infusion broth or on meat-infusion broth enriched with blood. It is of interest that the species can be differentiated on the basis of susceptibility to the bacteriostatic action of certain dyes. The growth of *B. abortus* is inhibited by thionine and that of *B. suis* by basic fuchsin, while *B. melitensis* is not inhibited by the same concentrations of the two dyes. The three species possess two antigens in common, the relative amounts of each varying with the species.

Since brucellosis occurs in areas where infectious abortion of cattle or hogs is endemic or where goats are infected, the best method of control is to eliminate the infection from the animal herds. This is not always easy to accomplish, and the easiest way to reduce greatly the incidence of the disease in man is to pasteurize all milk before it is used for drinking purposes or converted into dairy products. Occasional cases will occur in animal handlers, since the organism can be transmitted by direct contact as well as in milk. The brucellae are somewhat resistant to the sulfa drugs, but streptomycin and the tetracyclines are of value as therapeutic agents. They are difficult to eradicate because of their intracellular location.

### THE RESPIRATORY GROUP

The moist, warm mucous membranes of the mouth and throat support an abundant and varied population of microorganisms, numerous species of cocci, rods, and spirilla as well as yeasts, molds, and protozoa having been isolated therefrom. Some of these organisms were no doubt contaminants, having gained entrance in food or drink, and would not become established in the competition for existence in this area. Many are normal inhabitants, and a few are pathogenic species which, unless they gain entrance to, and establish themselves in, deeper tissues, live a primarily parasitic mode of life.

Both aerobic and anaerobic species are present on the gums and between the teeth. One prevalent concept is that the acid-producing bacteria, particularly the lactobacilli, around the teeth produce sufficient acid to



out through the enamel, exposing the dentine layer. Other bacteria may be involved in further decay of the teeth. Bacteria also gain entrance into the gums and at times establish foci of infection or abscesses around the root, or in the root canal, of a tooth. At times a more generalized infection is established with chronic inflammation about the roots of the teeth and with damage to the bone structure, the teeth eventually becoming loose in their sockets. This infection, which may be caused by different species of bacteria, is known as pyorrhea. At times a mixed population, consisting of spirochetes, fusiform bacilli (long, slender, tapering, gram-negative rods), vibrios, and cocci, becomes established in an apparently symbiotic relationship. A redness or congestion develops in the entire oral cavity, giving rise to a condition commonly known as trench mouth. A more common form of this fusospirochetal symbiotic infection develops on the gums or tonsils, giving rise to the condition known as Vincent's angina.

Other bacteria, particularly streptococci and staphylococci, may produce an infection of the tonsils known as tonsillitis. In other instances inflammation of the pharynx (pharyngitis, or just plain sore throat) develops as the result of invasion of the local tissues by bacteria, generally hemolytic streptococci. In a few instances, particularly in children, yeast-like fungi (*Candida albicans* or *Geotrichum candidum*) give rise to an infection in the mouth known as thrush. At times localized lesions caused by specific microorganisms develop in the tissues of the oral cavity, e.g., a primary syphilitic lesion. From such a lesion the organisms may spread to other parts of the body, giving rise to a more generalized infection. The streptococci and the diphtheria bacillus illustrate the pathogens which give rise to specific infections in the mouth and throat and which may spread or produce marked effect in other parts of the body.

**The Streptococci.** The genus *Streptococcus* of the family Lactobacteriaceae consists of many varieties of gram-positive, spherical bacteria that grow characteristically in chains. The streptococci are rather fastidious in their growth requirements and do not grow as readily, or remain viable as long, as coliform bacteria or the staphylococci. They can be roughly divided into two subgroups, the saprophytes and the parasites. The lactic acid streptococci of milk and the enterococci are the common members of the first group, while the hemolytic (beta type) and viridans (alpha hemolytic) groups of streptococci contain the pathogenic species. By means of agglutination and precipitation tests devised by Lancefield, Griffith, and others, the hemolytic streptococci can be divided into at least nine groups (A through K, omitting I) with a number of types, particularly in group A which contains most of the species pathogenic for man.



The alpha, or viridans, type of streptococcus, those which produce a brownish-green discoloration on blood agar, are primarily opportunists of low virulence and are therefore more commonly found in localized or mild infections. *S. salivarius*, an alpha-hemolytic species, does, however, appear to be responsible for practically all cases of subacute bacterial endocarditis. The beta-hemolytic streptococci, those whose colonies on blood agar are surrounded by a clear zone in which hemolysis is complete, contain the more important human pathogens. These streptococci were given species rank in earlier classifications on the basis of infections produced but now are considered as various types of one species, *Streptococcus pyogenes*. The following diseases are commonly ascribed to the various types of *S. pyogenes* comprising group A of the Lancefield classification:

Infections of teeth, tonsils, and sinuses  
 Infections of wounds  
 Erysipelas, an inflammatory disease of the skin  
 Puerperal sepsis, or childbed fever  
 Bronchopneumonia  
 Septic sore throat  
 Scarlet fever  
 Rheumatic fever

Streptococci are so versatile in their attacks that the same strain may give rise to different types of infection in different individuals, e.g., in one person to septic sore throat, in another to scarlet fever. The resistance of the host, including both general factors and immune bodies, does, however, influence the nature of the infection produced.

**Septic Sore Throat.** Epidemics of septic sore throat are frequently milk-borne, the streptococci coming either from milk handlers or from healthy carriers. Since the streptococci can grow readily in milk, the contaminated milk may serve as a source both of large numbers of bacteria and of the toxins which the streptococci produce. At least five exotoxins or toxic manifestations of cell-free filtrates can be demonstrated: hemolysin; leucocidin, which destroys leucocytes; fibrinolysin, which lyses fibrin clots; hyaluronidase, or the spreading-factor enzyme, which may facilitate spreading; and the erythrogenic, or skin-reddening, factor. Some streptococci may also produce a lethal factor, apparently not a true toxin. The streptococcus of septic sore throat is of the same type as the type responsible for scarlet fever.

**Scarlet Fever.** This disease is primarily one of childhood and, like other streptococcal infections, may vary markedly from mild to very severe cases in different individuals. A scarlet-fever strain of *S. pyogenes* becomes established in the throat and forms exotoxins which, when absorbed into the blood, cause the fever and skin rash of scarlet fever. In this respect the disease is much like diphtheria, the organisms tending

to remain localized in the throat while their toxin poisons the whole body. One attack generally confers a lasting immunity against scarlet-fever toxin but not against the causative streptococcus itself, hence an individual can develop repeated infections of the throat (septic sore throat) by the same strain of *S. pyogenes*, no lasting immunity being developed against the organism itself. It is possible to immunize man against the scarlet-fever toxin, but for best results the toxin itself rather than a toxoid must be used. For this reason immunization is employed only in individuals frequently exposed to the disease. The Dick test, mentioned in Chap. 21, is useful in determining resistance or susceptibility to scarlet-fever toxin. A reaction at the site of injection of a minute amount of the toxin indicates lack of antibodies capable of neutralizing the toxin. Penicillin is of most value in the control of scarlet fever and other strains of the group A,  $\beta$ -hemolytic streptococci, and has markedly reduced the severity of, and fatalities from, streptococcus infections. Antibiotic sensitivity tests should be the guide for antibiotics against other streptococci.

**Pneumonia.** By pneumonia is meant an inflammation of the lungs, occasionally caused by *S. pyogenes*, *Micrococcus pyogenes*, *Klebsiella pneumoniae*, *Hemophilus influenzae*, or other bacteria, but most commonly the result of infection produced by the pneumococcus, *Diplococcus pneumoniae*. The pneumococcus (see Figs. 3-6 and 20-21) is a fairly large, gram-positive coccus, somewhat lance-shaped in appearance and generally encapsulated, only the encapsulated strains being virulent. The pneumococci are closely related to the streptococci in their nutritional requirements and are alpha-hemolytic on blood agar but differ in their appearance in smears, the pneumococci generally occurring in pairs rather than in chains. A fundamental difference between the pneumococci and the streptococci is that the former are soluble in bile while the latter are not, this behavior being employed as a rapid method for distinguishing between the two genera in the laboratory.

The general course of pneumococcus pneumonia was described in Chap. 20 and will not be discussed further. It is one of the more common infectious diseases of man, is spread primarily by droplet infection, and no lasting immunity is developed against the pneumococci as a group



FIG. 23-2. Gram stain of *Streptococcus pyogenes*.

nor against the specific type involved in a pneumococcal infection. The organism is frequently found in normal throats and tends to invade the lungs when local or general resistance of the individual is lowered. The infection does not tend to spread to other parts of the body to the extent commonly observed with the streptococci, but pneumococci can be recovered at times from the blood stream. Like the streptococci, and the cocci in general, *D. pneumoniae* is susceptible to penicillin, other antibiotics, and the sulfa drugs.

**Meningitis.** Meningitis is an inflammation of the meninges, the membranes which cover the brain and spinal cord, and is commonly caused by *D. pneumoniae*, *S. pyogenes*, *Hemophilus influenzae* (the "influenza" bacillus), or the meningococcus, *Neisseria meningitidis*. The latter organism is the cause of the severe epidemics of cerebrospinal meningitis. *N. meningitidis* is a small, coffee-bean-shaped, gram-negative diplococcus which is strictly parasitic in habit and quite exacting in its growth requirements. Many individuals are carriers of the meningococcus, and it is difficult to explain why the species at times apparently becomes invasive and then tends to localize in the meninges. The cells probably set up a local inflammation in the nasopharynx, invade the blood stream, and finally become established in the cerebrospinal tract. Sometimes the infection is primarily one of the blood stream alone, and a typical bacteremia is the result, a rash developing in severe cases and giving rise to the name spotted fever for this type of infection. One of the mysteries of medical bacteriology is the tendency for most pathogens to localize selectively in organs or tissues in the body, tissues upon or in which they are not normally parasitic.

The meningococcus and the closely related gonococcus differ from the other pathogenic cocci in being gram negative in staining characteristics. Fortunately for man, these cocci are more susceptible than most gram-negative bacteria to the sulfa drugs and to antibiotics, although they do tend to develop resistance against these agents quite readily. One strange fact is that some streptomycin-resistant strains have been isolated from laboratory cultures and will grow only when streptomycin is present in the culture medium. Other gram-negative diplococci are commonly found in the mouth and on mucous membranes, and these parasitic but generally nonpathogenic *Neisseria* are somewhat difficult to differentiate from the pathogenic species. In general, the differentiation is made on the ability of the nonpathogens to grow more readily on ordinary media, on the basis of sugars fermented, according to the type of colonies produced, and on the basis of specific agglutination tests. Pathogenicity may be associated with the possession of specific antigenic components, but this alone does not suffice to explain why one organism is able to invade the

deeper tissues while closely related species are harmless parasites. This, as was also emphasized above, remains as one of the most puzzling problems in the microbiology of infectious disease.

**Diphtheria.** Diphtheria rather closely resembles scarlet fever in that many of the symptoms of the disease are the result of the action of an exotoxin produced by the organism, which tends to remain localized in the throat. Diphtheria was formerly one of the most dreaded of the infectious and contagious diseases, but it has been brought under control as the result of the development of an effective immunization procedure. Immunization is directed against the exotoxin and not against the diphtheria bacillus, *Corynebacterium diphtheriae*. This is one of the few pathogenic bacteria which can be recognized fairly readily by its appearance in stained preparations under the microscope. *C. diphtheriae* (see Fig. 2-10) is a relatively long, slender, nonmotile, nonencapsulated, gram-positive rod which is rather pleomorphic in form. The rods are frequently slightly curved and irregular, often with clubbed ends, and tend to stain irregularly with dye solutions such as Loeffler's methylene blue. Metachromatic granules are generally evident, and barring is frequently prominent, the cells appearing to be transversed by bands or bars which do not stain readily. This appearance may be the result of a multicellular state as suggested by Bisset. At times the organisms appear to branch, and for this reason they, together with the *Mycobacterium*, were classified with the Actinomyceetales. In the sixth edition of Bergey's Manual the diphtheria bacteria were placed in a separate family, the Corynebacteriaceae, under the Eubacterineae. The diphtheria bacilli are less exacting than the streptococci and meningococci in their growth requirements but do grow more readily upon enriched media than upon plain nutrient agar. Loeffler's medium, a glucose-infusion broth in coagulated beef serum, serves as an excellent menstruum for the growth of this bacterium and is frequently employed for its isolation from suspected cases or carriers. A blood-agar medium containing tellurite is also employed for the isolation of the diphtheria organism, the tellurite inhibiting the growth of some bacteria present in the nose or throat. *C. diphtheriae* reduces tellurite and as a result of this reaction produces characteristic dark-gray or black colonies on tellurite medium.

Diphtheria-like bacteria, called diphtheroids, are frequently present in normal throats but have no practical significance except that they can be confused with the diphtheria bacillus. As a rule, the nonpathogenic species, *C. pseudodiphthericum* and *C. xerosis*, are not as slender as *C. diphtheriae* and show less tendency to form metachromatic granules or to stain unevenly. They can also be differentiated on the basis of fermentation reactions, *C. xerosis* fermenting both glucose and sucrose with



acid production, *C. diphtheriae* fermenting glucose only, and *C. pseudodiphthericum* being a nonfermentative organism. Two other genera are recognized in the family Corynebacteriaceae, *Erysipelothrix* and *Listeria*, but are of limited pathogenic importance. *E. rhusopathiae* is the cause of swine erysipelas and *L. monocytogenes* of a disease of rabbits in which there is a great increase in the numbers of monocytes (mononuclear leucocytes). The latter organism is at times the cause of infection in man.

Diphtheria is an acute infection of the throat and is accompanied by

a severe toxemia, elicited by the potent diphtheria exotoxin. Severity of the disease can be greatly reduced by the therapeutic use of antitoxin, which is generally prepared in horses. The organisms give rise to an inflammation of the mucous membranes in the throat. Fibrin is exuded from the affected tissues and forms an adherent white coagulum, known as a "false membrane," which is rather characteristic of diphtheria infection. Since similar "membranes" are present in other infections of the throat, it is necessary to demonstrate the presence of diphtheria bacteria in smears or cultures made from the membrane.



FIG. 23-3. Emil von Behring, discoverer of antitoxin against diphtheria toxin.

Immunity to diphtheria can be determined in man by means of the Schick test, in which a minute amount of the diphtheria toxin is injected into the skin. The toxin is neutralized by antitoxin in the blood of immune individuals, nonimmune individuals exhibiting an inflammation at the site of the injection. Children should be immunized at an early age against diphtheria toxin, immunity being established following the injections of small amounts of formaldehyde-detoxified toxin (toxoid), which is generally purified to some extent and at the same time made more reactive by precipitation with alum. The use of synthetic media rather than complex broths for the production of diphtheria toxin has also led to the production of purer toxins and toxoids.

In the past few years there has been considerable increase in knowledge concerning the exotoxin of diphtheria. It appears to be the protein constituent of an iron-containing respiratory enzyme, the toxin apparently interfering with a metal-containing enzyme in the host. The reaction

occurs primarily in certain nerves and heart muscle, and the inference is that these tissues may be rich in the particular enzyme or enzymes concerned in the reaction. It was observed that the amount of toxin production is in part a function of the amount of iron in the culture medium, maximum toxin production occurring at an iron concentration below that which supported maximum growth of the diphtheria bacterium. In an iron-deficient medium, the cells synthesize porphyrin and toxin, but since iron sufficient to unite all of the protein toxin and the porphyrin is not present, free porphyrin and free toxin escape into the medium. When iron is present in quantities sufficient to support maximum growth, it is taken up by the cells, and more of the toxin-porphyrin complex is formed. This results in a diminution in the amount of toxin excreted into the medium. Pappenheimer has concluded from studies of this nature that diphtheria toxin is the protein moiety of an iron-containing respiratory enzyme present in *C. diphtheriae*. Fundamental studies of this type provide information concerning the nature and mode of action of substances associated with the virulence of pathogenic bacteria and will lead in time to a fuller understanding of the nature of infectious diseases. They also support the concept earlier advanced (Chap. 20) that pathogenic microorganisms have not evolved with intent to do damage to their host and that instead pathogenicity is more an evidence of bungling on the part of the parasite, certain constituents of cellular substance being toxic for certain other types of cells.

**Tuberculosis.** Tuberculosis is generally an infection of the lungs (pulmonary tuberculosis), but the organism, *Mycobacterium tuberculosis*, can and frequently does produce infection in the lymph glands, bones and joints, skin, or in fact in almost any part of the body. This bacterium is not a true bacterium of the suborder Eubacterineae but instead is classified in the order Actinomycetales. Actually the tubercle bacilli have much in common with the true bacteria, but they do form a transitional group between the Corynebacteriaceae and the actinomycetes, both in morphology and in staining reactions. The mycobacteria are characterized in part by the fact that they are acid fast in their staining properties, a property also shared by some of the actinomycetes. A number of saprophytic species are known, the important species pathogenic for man being *M. lepre* (the leprosy bacillus) and *M. tuberculosis*. The mycobacteria tend to be long, slender, slightly curved, gram-positive rods which frequently stain unevenly. They are not motile and do not form spores or capsules. In smears they frequently bunch together in groupings much like bundles of sticks. Most species are not particularly exacting in their growth requirements (particularly true of *M. tuberculosis*), but many tend to grow very slowly on laboratory media. They frequently show dull wrinkled colonies on agar and tend to form pellicles

on liquid media, the latter partly because of their high fat or wax content and their aerobic type of metabolism.

There are four distinct types of tubercle bacilli, the human and bovine types being included as varieties *hominis* and *bovis* in the species *M. tuberculosis*, while the avian (bird) and cold-blooded types are given species rank. *M. avium* is the species infective for birds. A number of species are parasites on cold-blooded animals such as fishes, turtles, snakes, and frogs, in which they produce a disease resembling tuberculosis. Man is subject to infection with either the human or bovine varieties, the former being the main cause of pulmonary tuberculosis, while the latter is more frequently involved in primary abdominal tuberculosis or in infections of the bones, joints, or lymph glands. The human variety is commonly spread by means of droplet infection, the bovine variety in milk from infected cattle. The spread of the latter type can be greatly reduced by the eradication of tuberculous cattle and by the pasteurization of milk, time and temperature of pasteurization being based on the relationship between these two factors in the lethal effect of heat on the tubercle bacillus.

The growth of tubercle bacilli within the tissues gives rise to the formation of characteristic nodules called *tubercles*. These arise from collections of phagocytic cells containing tubercle bacilli in the living state. In time the host develops a hypersensitivity to constituents of the bacterium, resulting in a marked reaction between the tissue cells and the bacteria. The body defenses attempt to wall off the infected areas with the formation of a thick envelope of fibrous tissue. As the struggle continues between the host and the parasite, the tubercle gradually enlarges, and cells in the interior die, giving rise to a soft, cheesy mass. If the host gains advantage, lime salts are deposited in this cheesy mass, and a firm wall of scar tissue is deposited around the entire mass. Tubercle bacilli may remain viable in tubercles for many years and later spread to other parts of the body. These calcified tubercles are observed in a large percentage of individuals in post-mortem examinations, indicating that a high proportion of apparently nontuberculous individuals had been subject to tuberculous infection at some time during their lives.

In the lungs or other organs, many tubercles appear when the organisms are active; the tubercles enlarge and coalesce, and destroy the normal tissues. In many instances much of the normal tissue may be consumed, and cavities appear in the affected organs, hence the popular term *consumption*, which so well describes a severe infection. The struggle between the host and the parasite is frequently a long one, the bacterium multiplying slowly but steadily and being fairly skilled in maintaining itself in comparison with most pathogenic species. Adequate rest and a well-balanced diet greatly aid the host in its struggle and, in



fact, are also protective measures against tuberculosis. This disease is most prevalent in undernourished, poorly housed populations and tends to flare up when conditions such as war or major economic depressions lower the living standards of man.

X-ray examinations are of value in the detection of tuberculosis, and diagnosis can be made on finding tubercle bacilli in smears or cultures made from sputum, gastric washings, urine, or other suspected material. The tubercle bacilli are sensitive to streptomycin and isonicotinic acid hydrazide, a combination of the two drugs being more effective than either alone, particularly because there is much less chance for drug-resistant strains to develop. Tuberculosis is one of the major infectious diseases of man today, over 80,000 new cases being reported annually in the United States along with around 15,000 deaths per year. Vaccines, particularly BCG, appear to have some value, but effective ones are rather difficult to prepare. The most effective control measures involve prevention of spread directly from infected persons or through milk from infected animals. The tuberculin test is of value in demonstrating the lack of resistance (susceptibility) to tuberculosis, but a positive test is not diagnostic since it indicates a hypersensitivity resulting either from past or present infection or even exposure to the organisms.

**Whooping Cough.** This disease is the result of a heavy infection of the bronchial tract with *Hemophilus pertussis*, a member of the family of small bacteria, the Parvobacteriaceae. The hemophilic, or blood-loving, bacteria are a group of small, gram-negative rods characterized by the fact that they grow most readily when blood is added to nutrient agar, blood supplying essential growth factors. *H. pertussis* is a short, oval, gram-negative rod with a tendency to exhibit bipolar staining. Smooth, virulent strains are encapsulated. It is an aerobic organism of low metabolic activity as compared with most bacteria and is a strict parasite.

Whooping cough begins much like an ordinary cold, but in a period of 7 to 10 days the characteristic "whoop" develops, being caused by the effort of the infected individual to get his breath after a fit of coughing. The organisms are present in large numbers in the droplets expelled during coughing and can be demonstrated by means of a "cough plate." This is made by holding a glycerol-potato-blood agar plate open in front of the mouth during a coughing spell. The organism of whooping cough develops characteristic, small colonies on this medium on incubation for 24 to 48 hr. at 37°C. The pertussis bacteria have no marked poisonous properties, the symptoms of the disease commonly being caused by the very heavy growth of the organism on the mucous membranes of the throat and in particular of the bronchi and bronchioles. The bacterium has little or no invasive powers but, particularly in young children, may



give rise to a bronchopneumonia. Most adults are immune, the immunity being due primarily to their having had the infection during childhood. Immunization with killed bacteria or with mechanically disrupted cells is of value in the prevention of the disease in children, or in greatly reducing the severity of the infection.

The vaccine commonly is given in conjunction with tetanus and diphtheria toxoids. Hyperimmune globulin injected early in the course of the infection reduces the severity of the disease and can also be used as a passive prophylactic measure. *H. pertussis* is susceptible to many of the antibiotics, which do tend to shorten the course of the disease. Their major value, however, is that they diminish the incidence of secondary infections and of pneumonia.

**Virus Infections: The Common Cold and Influenza.** A number of infectious diseases of the respiratory tract are caused by filtrable viruses and organisms such as *Mycoplasma psittaci* (commonly spoken of as psittacosis virus) on the border line between the smaller viruses and the bacteria. Still other viruses enter the body by way of the respiratory tract but commonly invade deeper tissues, the virus of poliomyelitis serving as an example of this group. These viruses leave the body by way of the respiratory tract, or they may be transmitted from skin lesions or in excreta and may possibly gain entrance to the body by routes other than the respiratory tract. The latter, however, appears to be the most common portal of entrance, and such viruses as mumps and poliomyelitis are arbitrarily considered here.

The virus or viruses that cause the common cold constitute one of the most important plagues of man. Little is known concerning the agent involved; much of the evidence for its being a filtrable virus is of indirect nature, although recent studies quite definitely indicate that it is a small virus. The typical "cold" develops rapidly, after an incubation period of 24 to 48 hr., with congestion of the nasopharynx and copious watery discharges from nasal and conjunctival mucous membranes. This watery discharge persists for 24 to 48 hr., being replaced by a thick discharge containing large numbers of those bacteria commonly present in the nose and throat. Infection with the virus apparently weakens local tissue resistance to such an extent that the opportunists can invade local tissues, multiply, and elicit a marked inflammatory response. In fact many of the symptoms of a common cold can be caused by a mixture of bacteria, and it is difficult to differentiate clinically between a viral cold, an acute bacterial infection of the upper respiratory tract, and a mild case of influenza. No lasting immunity is built up against the cold virus, most individuals having two to three colds a year. This suggests the existence of a number of cold viruses varying in their antigenic structure, but proof of this concept is lacking. Colds commonly pave the way for invasion

of the lungs by other bacteria, and pneumonia is at times a result of the activity of secondary invaders. The cold virus in particular appears to be able to upset readily the normal ecological balance in the upper respiratory area, although fatigue, chilling, emotional upsets, and other factors can also do so, paving the way for enhanced bacterial growth and cold-like symptoms. This suggests the complicated relationships influencing the normal balance between a parasite and its host and the result of a slight change in this biological balance of forces.

It has been definitely established that influenza is induced by a virus. Three distinct types, A, B, and C, are recognized, and there is evidence that there are other types or subtypes. The influenza virus is spherical to bean-shaped, with a diameter near 100  $m\mu$ . Electron micrographs suggest that these viruses are organized bodies surrounded by a limiting membrane, but they have never been cultivated away from living tissues. A "soluble" particle around 10  $m\mu$  in diameter is associated with multiplication of the influenza viruses, but the soluble substance is not infectious by itself.

Influenza virus invades and destroys epithelium in the upper respiratory tract, and the disease is characterized by greater debility of an infected individual than is noted with the common cold. Uncomplicated influenza is a relatively mild disease with few respiratory symptoms unless accompanied by secondary invasion by cocci or by *Hemophilus influenzae*, once thought to be the bacterium causing influenza in man. Immunization with treated preparations of the prevalent type of the virus cultivated on the chorioallantoic membrane of chick embryos is of transient value in the control of influenza. The virus tends to alter in its antigenic structure quite readily and, therefore, the available vaccines may offer little or no protection against the new type or subtype.

Among military recruits within the United States during the Second World War, an acute pneumonitis was recognized as being caused by a virus, and the disease was designated primary atypical pneumonia. Since then a number of similar viruses have been recognized. The viruses are commonly found in adenoid and tonsil tissues, give rise to mild respiratory infections or febrile catarrhs, are also causative agents for a conjunctivitis, and are noninfectious for laboratory animals but will multiply in tissue cultures. The viruses all appear to be around 90  $m\mu$  in diameter and are commonly spoken of as the acute respiratory-disease (ARD) or adeno-virus group. Eleven or more antigenic types have been recognized, type 4 being responsible for the primary atypical pneumonia mentioned above.

Another group of viruses, the Coxsackie group, causes fever and sore throat, among other symptoms, and often the causative virus can be isolated from throat washings. Later on during the course of the illness, the virus may be found at times in the blood and more commonly in

stools. At least 24 antigenic types have been recognized. They appear to fall into two distinct groups (A and B), and are relatively small viruses, approximately 25 to 35  $m\mu$  in diameter. They cause a variety of minor illnesses, some of which (summer minor illnesses) occur primarily during the warmer months of the year. These viruses are recognized most readily by the symptoms they induce in newborn mice and by serological tests. These viruses appear to be rather widespread and are of minor importance in that few or no fatalities have been attributed to infection with viruses of the Coxsackie group.

Other viruses are recognized from time to time, e.g., the enteric cytopathogenic human orphan (ECHO) viruses. These viruses may cause an aseptic meningitis like that induced by the Coxsackie viruses, but the two groups are distinct from each other. They have been isolated at times from children suspected of having nonparalytic poliomyelitis.

**Measles.** Measles (rubeola) is one of the most infectious specific infections of man. Most cases appear in children, the majority of adults having had the disease during their childhood and having developed a relatively permanent resistance to the infectious agent. The causative agent is a filtrable virus about which relatively little is known. It is present in the blood stream and respiratory secretions early in the course of the disease and in the skin rash which develops after the respiratory symptoms have appeared. Measles by itself is not a very severe disease, but, like influenza, it appears to lower resistance to the gram-positive cocci and to the influenza bacillus. Gamma globulin is of value both as a prophylactic and as a therapeutic measure. German measles (rubella), an infection somewhat related to measles, is a milder disease and, like measles, is a disease primarily of childhood. Rubella, however, during the first few months of pregnancy, can induce abnormalities of the fetus. A high degree of immunity is developed but not of the grade observed with true measles, since reinfection does occur in some individuals.

**Psittacosis.** This is a disease naturally occurring in birds which is readily transmitted to man from infected birds, or occasionally from man to man. It is caused by one of the larger filter-passing agents, between 200 and 300  $m\mu$  in diameter. The virus bodies can be seen as faintly staining coccid or bacillary forms in appropriately prepared smears. They appear to multiply by binary fission and to pass through a cycle of development involving globoid bodies several microns in diameter. Upon rupture of the globoid body a large number of the elementary forms are released, and these are infectious for other cells. The virus appears to be susceptible to large doses of penicillin or the tetracyclines, another factor suggesting its close relationship with the bacteria.

**Mumps.** Mumps is characterized by an inflammation and swelling of the salivary glands, although the infection can involve other glands.



particularly the reproductive glands in the adult male. The virus is relatively large, electron micrographs suggesting a coccal form near  $170\text{ m}\mu$  in diameter. Little is known about the nature of this agent. It has been successfully cultivated in the developing egg. An attack of the disease confers a relatively lasting immunity, and attempts are being made to develop a vaccine. The use of immune serum of high titer appears to have some value as a prophylactic measure and also to some extent therapeutically, in particular inhibiting the spread of the virus to the testes in the male.

**Poliomyelitis.** This disease, commonly known as infantile paralysis, is one of the dreaded infections of man because of the more or less permanent damage which can be produced in the limbs as the result of nerve and muscle impairment. Actually, the incidence of the crippling infection is relatively low when compared with the common childhood diseases such as measles, mumps, and chicken pox. It is believed that the virus enters the body by way of the respiratory or digestive tract and may remain there, giving rise to nonrecognized cases; or it may enter nerve cells and migrate to the brain and spinal cord with or without marked production of paralysis of different groups of muscle.

Poliomyelitis virus is one of the smaller filter-passing agents, being about  $30\text{ m}\mu$  in diameter (see Fig. 7-11). Relatively little is known concerning the actual nature of this agent, although it has been obtained in highly purified form and appears to be a nucleoprotein. Certain strains of the virus have been cultivated in the past few years in the developing hen's egg and in embryonic tissue growing *in vitro*. No effective therapeutic agent has been found to prevent the development of the infection once it is recognized, but the Salk vaccine, prepared by formaldehyde inactivation of the three major types of the virus grown in monkey kidney tissue culture (see Figs. 7-9 and 7-10), appears to be effective as a prophylactic measure; mass immunization of the younger population and pregnant mothers has been attempted.

**Miscellaneous Infections.** We have considered the more important pathogenic agents which produce infections of the respiratory tract or which commonly enter the body by way of this tract. Other organisms do at times cause respiratory infections. Infections of the lungs can be caused by bacteria other than those mentioned here. For examples, pneumonic plague and woolsorter's disease could suffice. The Black Death, or bubonic plague, was formerly one of the great scourges of mankind. This is generally an infection of various glands or lymph nodes by *Pasteurella pestis*, but this bacterium does at times produce an infection of the respiratory tract, pneumonic plague, which is highly fatal. *Bacillus anthracis* generally produces localized skin infections or a bacteremia in man, but the spores may be inhaled and give rise to a highly fatal in-



fection of the lungs. This is most frequent amongst handlers of wool or of hides obtained from animals in areas where anthrax is prevalent, hence the name woolsorter's disease. Lung infections may also be caused by species of the higher fungi, particularly from the genera *Actinomyces*, *Aspergillus*, and *Blastomyces*.

The yeast-like organism *Coccidioides immitis* is of considerable biological interest in that it resembles yeasts or *Blastomyces* in the tissues, while the growth on culture media is similar to that of typical molds. *C. immitis* does not bud in the tissues but develops spherical, thick-walled

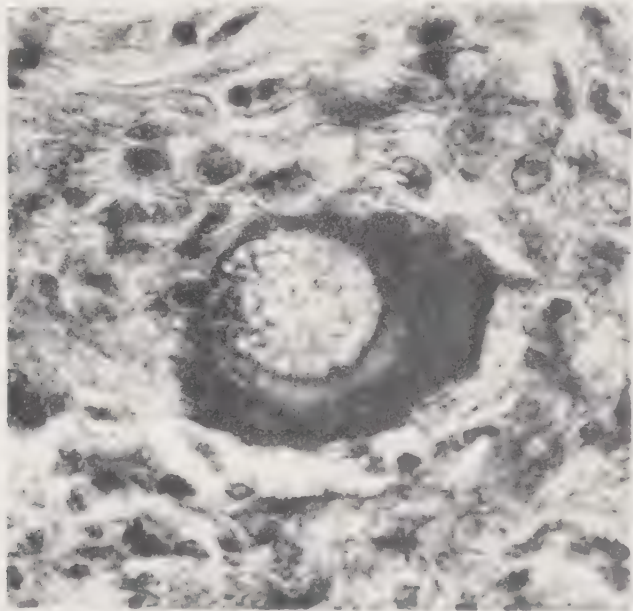


Fig. 23-4. Endospores in a sporangium of *Coccidioides immitis* in body tissue. (Courtesy of Charles Smith.)

bodies 15 to 80  $\mu$  in diameter (see Fig. 23-4). These cells, when mature, are filled with small endospores 2 to 5  $\mu$  in diameter. When the spherules rupture, the spores are disseminated and can give rise to the development of new spherules; the immature cells containing no endospores and in appearance closely resembling *Blastomyces dermatidis*. On Sabouraud's agar a flat, membranous colony develops and becomes covered with an aerial mycelium, cottony in appearance. These mycelial growths break up into numerous thick-walled arthrospores (see Fig. 23-5) which are readily dislodged and frequently spread the infection in the laboratory.

Coccidioidomycosis is probably a dust-borne infection and is most common in the San Joaquin Valley in California and in other semidesert areas of the southwestern United States. It commonly causes a benign pulmonary infection known as Valley fever, the disease closely resembling

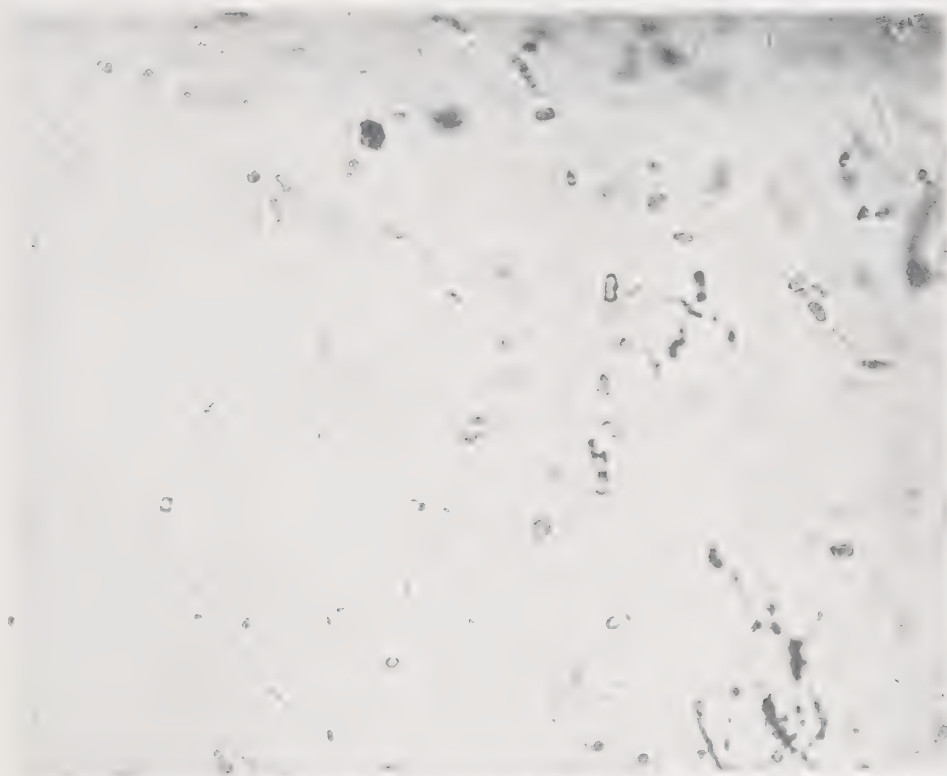


FIG. 24-5. Chlamydozooids and arthrozooids of *Coccidioides immitis* broken away from the mycelium in a culture. (Courtesy of Charles Smith.)

**Influenza**—The organism can develop in other tissues, and when it spreads throughout the body, it gives rise to a highly fatal infection.

### INTIMATE-CONTACT GROUP

Abrasions or wounds in the skin, mucous membranes, or deeper tissues pave the way for the entrance of bacteria and other parasites which, if they become established, alter a state of contamination to one of infection. Staphylococci and streptococci are commonly associated with man and frequently are responsible for localized infections in the skin and mucous membranes. The general picture of this type of infection was developed in Chap. 20 and will not be considered further. These organisms can also cause infections in deeper wounds and can spread throughout the body. The enteric group, particularly the coliform bacteria, are often responsible for infections of wounds in the abdominal or peritoneal cavities. *Pseudomonas aeruginosa* is also frequently found in wounds, generally as a secondary invader. It closely resembles the enteric group but can be readily differentiated in that it produces a blue-green pigment

mixture, this being responsible for the blue- or green-colored pus encountered with infections established by this organism. It is relatively non-virulent but commonly gains entrance to wounds and develops as a secondary invader. It is becoming of more importance at the present time; since penicillin therapy may overcome the common gram-positive cocci also present, the balance established between these species is upset, and the gram-negative, penicillin-resistant *P. aeruginosa* can continue to develop. The clostridia are, however, of more potential danger in wounds than the organisms mentioned above. The latter may pave the way for the development of the anaerobic, spore-bearing rods of clostridia by their consumption of oxygen and the establishment of anaerobic conditions, an example of metabiosis. The two most common anaerobic infections are tetanus and gas gangrene.

**Tetanus.** Tetanus is characterized by continuous contractions of the muscles, the muscles of the jaw being involved early in the course of the toxemia, this reaction being the source of the common name for the infection, lockjaw. *Clostridium tetani* (see Fig. 2-9) is a slender, gram-positive rod with a terminal spore which causes the rod to swell, giving it a drumstick or tennis-racket appearance. It is a common, nonpathogenic, parasitic inhabitant of the intestinal tract of horses and other herbivorous animals, is sometimes present in the intestinal contents of man, and the spores are widely disseminated in nature. The organism does not develop readily in the tissues but, once it is established, liberates an extremely potent exotoxin which is responsible for the symptoms of the disease. The organism itself remains localized in the tissues, but the toxin spreads throughout the body, attacking primarily the motor nerve centers and thereby eliciting the tetanic convulsions.

Tetanus toxin has been obtained in highly purified form, is proteinaceous in character, and of such a high degree of toxicity that 1 mg. of the toxin would suffice to kill approximately 10 million mice. Fortunately it is antigenic, and the antigenicity is maintained on treatment with formalin, which inactivates the poisonous properties. Tetanus toxoid is an excellent immunizing agent, and all individuals should be immunized with it as a protection against tetanus toxin. This is frequently done in childhood, a mixture of tetanus and diphtheria toxoids being employed in many instances for simultaneous immunization against both toxins. Antitoxin is of value as a therapeutic measure.

**Gas Gangrene.** Gas gangrene is a general term employed to denote infection of deep wounds by different species of the anaerobic bacilli, the infection being marked by the production of considerable quantities of gas in the wound. The bacillus most commonly associated with gas gangrene is *Clostridium perfringens*, a short, plump, nonmotile, encapsulated, gram-positive, spore-bearing rod somewhat less exacting of strict anaerobic



conditions than is *C. tetani*. Other anaerobic bacilli commonly found in infected wounds can be divided into two main metabolic groups, saccharolytic and proteolytic. The former group contains *C. septicum* and *C. novyi* along with *C. perfringens*, while *C. sporogenes*, *C. histolyticum*, and *C. lentoputrescens* are representative of the proteolytic species. The proteolytic species are true saprophytes in that they cannot initiate an infection by themselves, but they complicate established infections by their intense proteolytic action. Whether the saccharolytic species are pathogenic by themselves is a debated point, their activity in tissues generally being associated with growth of pyogenic cocci.

*C. perfringens* (*B. welchii*) infections and the toxemia produced can well illustrate gas gangrene. This organism, commonly present in the feces of man and animals, readily gains entrance to a wound along with other soil forms and organisms parasitic upon man. Once the bacillus is established in a deep wound, it ferments muscle sugar with the production of large quantities of gas. It is, therefore, frequently spoken of as the gas bacillus. Its gas-producing ability can be demonstrated readily in a tube of milk layered with oil or cream to hinder the diffusion of oxygen into the milk. Acid (lactic and butyric) production is marked, and the milk coagulates with curd and whey production. The acid clot is torn apart by the gas produced, and material is spattered around the tube, hence the name "stormy fermentation" for the appearance which is so characteristic of *C. perfringens* fermentations.

*C. perfringens* produces three or four major exotoxins, the most important of which is the alpha toxin. Macfarlane and Knight identified this toxin as an enzyme which hydrolyzes lecithin, a phosphorylated fat widely distributed in the body. Lecithinase, the  $\alpha$  toxin, is antigenic, and antitoxin produced against toxic filtrates of *C. perfringens* neutralizes this enzyme. A collagen-splitting enzyme, collagenase, is also present in the filtrates and is believed to be a fourth toxin, the kappa toxin. It is involved in the destruction of muscle fibers. We have already noted that diphtheria toxin is the protein moiety of a respiratory enzyme. These observations with *C. perfringens* toxins suggest that other toxins may also be enzymatic in character. These exoenzymes or exotoxins must be of some value to the bacteria, but, being able to attack important constituents of the body of man or other animals, they are of extreme danger to the individual once they are formed in, or gain entrance to, the circulating fluids.

Antitoxins can be produced against the toxins formed by the saccharolytic anaerobes *C. perfringens* and *C. novyi*, and toxoids have been developed that are of some value as immunizing agents. They can be combined with tetanus toxoid and the mixture used for the immunization of individuals who are apt to be exposed to extreme physical risks. Chemo-



therapy with penicillin, streptomycin, or the sulfa drugs is of value immediately after an individual has been injured, particularly in that it inhibits the pyogenic cocci, thus rendering establishment of the anaerobes more difficult. These agents are also inhibitory to the anaerobes themselves, but sometimes a higher blood level is required than against the cocci.

**The Venereal Infections.** A number of infections are characterized by the fact that the infectious agent is spread for the most part by means of sexual contact involving an infected individual. The four more common venereal infections are gonorrhea, chancreoid, syphilis, and lymphogranuloma venereum. The external genitalia normally support growth of a varied microbial flora, most of which are saprophytic but which may at times give rise to localized infections or invade the urinary tract. In the latter group, staphylococci, streptococci, coliform and related organisms, and enterococci are the most common causes of infections of the urinary tract and bladder, while the hemolytic streptococci (frequently introduced from external sources) are the main cause of puerperal, or childbirth, fever, an infection of the uterus. These organisms produce the nonspecific infections of the genitourinary tract.

**Gonorrhea.** It has been estimated that over a million cases of gonorrhea are acquired annually in the United States alone, the majority of these cases being acquired during sexual intercourse, although the infectious agent can be transmitted on materials soiled with discharges from lesions or during the passage of the child through an infected birth canal. In the latter case, the infection of the newborn is generally limited to the eyes and can be prevented by the application of a 2 per cent solution of

silver nitrate to the conjunctiva immediately after birth. The infection in its various forms is caused by the gonococcus, *Neisseria gonorrhoeae*.

*N. gonorrhoeae* (see Fig. 23-6) is a small, oval or spherical, gram-negative coccus which generally occurs in pairs, the adjacent sides being flattened and giving rise to a kidney-bean-shaped appearance. The gonococcus is indistinguishable from the other neisseriae in its general appearance and, like the meningococcus, is somewhat difficult to cultivate in the laboratory. It is strictly a parasite of man, not giving



FIG. 23-6 Gram stain of urethral pus showing gonococci within phagocytic cells.

rise to infection in other animals, and will frequently persist in man for years with little appreciable damage once the acute stage has been passed. The gonococcus is taken up readily by the phagocytic cells, and a diag-

tests of gonorrhea can be established with fair security upon finding typical gram-negative cocci in phagocytes from pus obtained from lesions of the genital tract. Complete identification would require isolation and identification of the organism. The gonococcus is quite sensitive to the sulfa drugs, to penicillin, and to streptomycin, but it does have a marked tendency to develop drug-fast strains; increased resistance to one agent, however, does not denote increased resistance to unrelated chemotherapeutic agents. No successful method of immunization has been developed, and it is unlikely that one will be, since the infection itself leaves no immunity to subsequent reinfection once the original infection has been overcome. When arthritis or other gonococcal infections of the internal organs have been established, complement-fixing antibodies do appear, generally in low titer, again indicating the poor value of the gonococcus as an immunizing agent.

**Chancroid.** This is a common infection of the external genitals, characterized by the development of numbers of small, painful ulcerations which somewhat resemble the primary syphilitic lesion but never harden as does the latter. The lesion is therefore called a soft chancre. It is caused by *Haemophilus ducreyi*, which is quite closely related to *H. influenzae*. Chancroid is spread primarily by sexual contacts but has no serious consequences. The infection can be controlled readily by prompt, local treatment with the sulfa drugs or appropriate antibiotics.

**Syphilis.** This venereal infection is caused by a spirochete rather than by a bacterium, the organism in some respects more closely resembling the protozoa than the bacteria. The causative agent, *Treponema* (formerly *Spirochaeta*) *pallidum*, is a strict parasite adapted to growth in the tissues of man and only to a limited extent in other animals. It is extremely difficult to cultivate in the laboratory, and most reports of cultivation in inanimate media have never been confirmed. *T. pallidum* is a long, slender, coiled organism (see Fig. 23-7), generally 8 to 14  $\mu$  in length by 0.25 to 0.3  $\mu$  in diameter, containing 8 to 14 regular rigid spirals. It is motile, electron micrographs suggesting the presence of flagella, but it does not move rapidly or far. It does not stain with the regular aniline dyes and can be most readily recognized by its appearance in dark-field preparations.

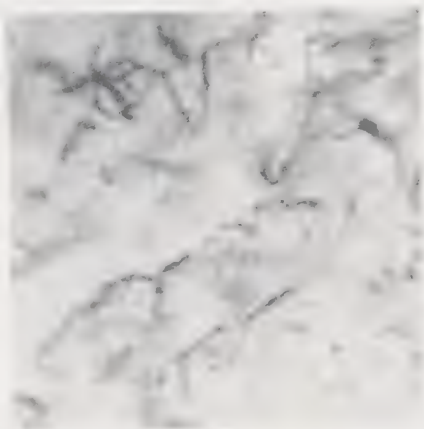


FIG. 23-7 *Treponema pallidum* in silver-impregnated stain of lung tissue.

Syphilis is spread almost entirely by sexual contact, since the organism cannot survive for any length of time outside the body. It differs from most of the infectious agents spread by intimate contact with infected or



FIG. 23-8. *Treponema pallidum* in a dark field (Courtesy of the American Optical Co.)

contaminated material in that it apparently has the ability to penetrate the unbroken skin or mucous membranes.

Syphilis in man progresses through a number of stages, the tertiary stage in particular being quite irregular in that any part of the body may be involved, the actual involvements varying markedly from person to person. In general, three distinct stages may be recognized, the important characteristics of which may be summarized as follows:



*The Primary Stage.* The initial lesion becomes evident after an incubation period of 3 to 6 weeks, enlarges, becomes hardened, and may ulcerate. In many cases the spirochetes can be demonstrated in fluid expressed from the chancre, the material being examined with the aid of dark-field microscopy or in silver-impregnated or otherwise stained smears.

*The Secondary Stage.* The spirochetes spread from the original site of the lesion and form secondary lesions in other parts of the body, generally on cutaneous and mucous membranes, but systemic infections can develop.

*The Tertiary Stage.* After the acute symptoms of the primary and secondary stages have passed, the organism remains more or less latent in the body, apparently a balance being established between the parasite and the host. After many months or years a shift in the balance takes place, and any part of the body can be involved in the disturbance. Cardiovascular and central nervous systems are most commonly involved, resulting in a variety of disorders including partial paralysis, insanity, and death.

After the early primary stage of the infection, diagnosis is commonly made on the basis of complement fixation or precipitation tests, described in Chap. 21. These tests become negative following successful treatment of the infection, a condition not observed in the common bacterial infections and suggesting that the serological tests (Wassermann or Kahn, for example) are based on something other than a true antigen-antibody reaction. A positive test is a sign of infection rather than of immunity in syphilis. The spirochete of syphilis is susceptible to mercurial and arsenical drugs and particularly to penicillin, but therapy has to be extended over a period of time or be intensively applied in order to obtain good results.

**Lymphogranuloma Venereum.** This is a virus infection of the genitalia characterized by ulceration and enlargement of the inguinal and pelvic lymph glands, although the virus may spread and give rise to a generalized infection. The elementary bodies are from 200 to 350  $m\mu$  in diameter, the larger forms being encapsulated. It has not been cultivated on lifeless media but develops in tissue cultures and in the fertile egg. It is closely related to the virus of psittacosis and has been placed with it and other closely related infectious agents in a proposed genus *Miyagawanella* as the type species, *M. lymphogranulomatis*. Lymphogranuloma venereum was considered to be a disease of tropical countries, but in recent years its prevalence in temperate regions has been recognized. It is not so serious a threat as syphilis or gonorrhea and can be controlled by penicillin or the sulfa drugs.

**Smallpox.** This was one of the dreaded diseases of early times and one that was spread readily from man to man. It is now almost stamped out in areas where vaccination is compulsory. This disease is of historical



importance in bacteriology since a method for immunization was developed against it long before the germ theory of disease was established. The Chinese had long known that local reactions frequently occurred when material from smallpox lesions was rubbed on the skin, and that the treated individual, if he did not develop generalized smallpox, became immune. Jenner, in 1798, reported his observations that dairy workers who had previously had cowpox appeared to be immune to smallpox. In 1796 he inoculated a boy with pus from a cowpox lesion and noted a local reaction. Two months later the boy was inoculated with pus from a smallpox lesion, and no infection developed. Immunization with cowpox rather than with smallpox material is much safer, and immunization against smallpox remains in principle the same as in Jenner's studies. Fortunately for man, the viruses of smallpox (*variola*) and of cowpox (*vaccinia*) are apparently identical antigenically or at least in their immunizing powers, it being suggested that *vaccinia* virus is a modified form of *variola* virus. *Vaccinia* virus is one of the larger viruses, electron micrographs indicating a size of 222 by 284 m $\mu$ . It appears to be relatively complex in chemical composition and to be approaching the complexity of true cells. This virus is produced in calves or in tissue cultures for the commercial production of vaccines. Vaccination confers a high degree of immunity for several years, but it should be repeated about every seven years if immunity is to be maintained.

**Fungi Transmitted by Contact.** A number of species of yeasts and molds are parasitic on the skin and mucous membranes, and they may invade the deeper tissues at times, although their power of invasion is low. They usually incite infections of the skin which are not particularly dangerous but are irritating and difficult to treat successfully. The main pathogenic species were listed in Chap. 5 and will not be considered further, since they are difficult to consider in limited space and present many complicated problems of special nature.

#### INFECTIONS SPREAD BY CONTACT WITH ANIMALS

Animals other than man have their characteristic parasites, a few of which are pathogenic to man. Some are transmitted directly through contact with the animal or its wastes, while others, which will be considered separately, are transmitted by means of an insect vector. We have already considered certain of these agents and the infections they produce when the causative agent tends to enter the body by a characteristic route. These organisms are the *brucella* group and the bovine type of the tubercle bacillus by way of milk, and the virus of psittacosis, the anthrax bacillus, and the plague bacterium via the respiratory tract. *Systemic* anthrax, glanders, tularemia, hemorrhagic jaundice, rat-bite

fever, and rabies exemplify infections which can pass from an infected animal to man, generally through an abrasion.

**Anthrax.** *Bacillus anthracis*, which causes a serious blood-stream infection in domestic animals, is of more historical interest than potential danger to man. The studies of Davaine, of Pasteur, and of Koch established the etiology of this disease and placed the concept of the microbial origin of infectious diseases on a firm foundation. *Bacillus anthracis* is a large, spore-bearing, aerobic, gram-positive rod characterized by the formation of a "Medusa's head" type of colony on nutrient agar. Man acquires the infection by direct contact with infected animals or products therefrom (particularly hides and wool), the spores entering an abrasion, germinating, and the vegetative cells giving rise to the production of an abscess known as a malignant pustule. The bacteria may spread from the pustule and produce a generalized infection, the blood stream teeming with the bacteria and death resulting from this severe bacteremia. Anthrax has been brought under control by immunization of domestic animals with killed suspensions of the anthrax bacillus, by avoiding heavily contaminated pasture lands, and by the careful disposal of the remains of animals dead from anthrax. Most antibiotics are active against the anthrax bacillus.

**Glanders.** Glanders is not a common infection of man but does occur in individuals who handle horses infected with the glanders bacillus, *Mullemyces mallei* of the Parvobacteriaceae. *M. mallei* is a slender, gram-negative rod, 0.5 to 1.0 by 2 to 5  $\mu$ , with rounded ends. There is a tendency for it to produce filaments and to show branching involution forms. The organisms exhibit irregular staining, frequently of a bipolar type. The bacterium enters the body through abrasions in the skin or mucous membranes and merites the formation of inflammatory nodules in the nose (glanders) or in the skin (farcy), and may spread throughout the body. The infection is generally fatal for man and can be prevented by exercising great care in the handling of infected animals and their nasal discharge. *M. mallei* is one of the most dangerous pathogens, and extreme care must be employed when working with it in the laboratory.

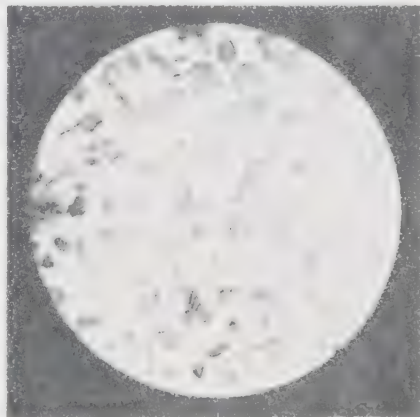


FIG. 23-9. An early photomicrograph of *Bacillus anthracis* in the blood of a guinea pig dead from anthrax. [From Roux, *Annales de l'Institut Pasteur*, 1, 209 (Fig. 2, Plate VII) (1887).]

**Tularemia.** This disease, also known as rabbit fever or deer-fly fever, is primarily one of wild rodents and rabbits. The infectious agent, *Pasteurella tularensis*, is spread by direct contact with the carcass of an infected animal, by inadequately cooked meat from infected animals, or by the bite of a contaminated insect such as the deer fly. The disease, which is neither severe nor very prevalent, is generally limited to hunters or to those who prepare wild rabbits for the table. The organism enters the skin through minute abrasions, some evidence suggesting that it can penetrate the intact skin. *P. tularensis*, a member of the Parvobacteriaceae, is a minute coccoid rod, 0.2 by 0.2 to 0.7  $\mu$ , which is extremely pleomorphic, gram negative, and with a tendency to bipolar staining. It does not grow readily except on highly enriched media, cystine in particular favoring growth.

**Hemorrhagic Jaundice.** This disease, also known as infectious or spirochetel jaundice, leptospirosis, or Weil's disease, is caused by the spirochetè *Leptospira icterohaemorrhagiae*, or by the closely related species *L. canicola*. The former is usually associated with wild rats or mice, the latter with dogs. The organisms are usually transmitted to man on contact with spirochetes present on materials contaminated with the urine of infected rodents or dogs. The disease is most common in workers in damp places. The leptospirae are microaerophilic, extremely slender, tightly coiled spirochetes with a hooked end or ends. They are extremely motile in dark-field preparations, showing flexing movements and coarse undulations. Diagnosis of the infection in the early stages is made by finding the characteristic leptospirae in dark-field preparations of blood, in the latter stages in urine, and also by injection of blood or urine into guinea pigs, which are highly susceptible.

**Rat-bite Fever.** This disease, which is not very common, is of interest in connection with leptospirosis in that it is primarily an infection of rats by a bacterium with certain characteristics of the spirochetes. The causative organism, *Spirillum minus*, is a gram-negative, spiral organism 3 to 5  $\mu$  in length with one to four spirals. It is more rigid than the spirochetes but, like many of the latter, is difficult to study in the laboratory as it cannot be cultivated readily, if at all, on laboratory media. It is transmitted to man in the wound made by the bite of an infected rat and produces a localized infection similar to that caused by *Streptobacillus moniliformis*.

**Rabies.** Rabies is an infection of dogs, wolves, and other carnivorous animals which can be transmitted to man as the result of the bite of an infected animal. In Brazil and Trinidad, rabies also appears to be an infection of vampire bats and can be transmitted by them. Infected bats are also found in the United States and are involved in the transmission



of rabies, particularly to cattle. The agent is one of the larger viruses, the particle being between 100 and 250  $m\mu$  in diameter. Rabies is of historical interest since it was the first virus disease to be studied in the laboratory and since Pasteur was able to develop a method of immunizing against the causative agent. Successful immunization after exposure is possible because of the long incubation period, 2 to 8 weeks or more, except when the bite is on the neck or face. The long incubation period when the bite is on the extremities supposedly results from the slow rate of travel of the virus to the central nervous system. This time interval provides opportunity for antibody production against the noninfective virus present in antirabies vaccines, and against the active virus as well. The immunization procedure is slow and irritating, hence it is not employed as a routine procedure with unexposed individuals.

### INFECTIONS SPREAD BY INSECTS

A number of infectious agents are commonly spread from animal reservoirs, or from man to man, by means of an intermediate insect vector. Bubonic plague serves as an example of a bacterial infection spread by insects; typhus and Rocky Mountain spotted fevers are rickettsial infections; yellow fever is of viral origin; and malaria is a protozoan infection.

**Bubonic Plague.** "Rats, Lice and History" is a fascinating book by Zinsser, who traces the influence of typhus fever, spread from rats to man by lice, upon the course of history. Bubonic plague, spread from rats to man by fleas, has also had considerable influence upon history, as it was one of the most dreaded epidemic diseases in earlier days. Plague, primarily a disease of rodents, is ordinarily transmitted from infected rats, upon death of the latter, by means of their contaminated flea population. When the flea bites man, the causative organism, *Pasteurella pestis*, enters the lesion and multiplies. It then invades the tissues, generally localizing in the lymph glands, which swell, later soften, and discharge pus. The characteristic, swollen lymph glands, particularly in the groin, are called buboes and gave rise to the name bubonic plague. The organisms escape from the lymph nodes and establish a generalized infection. A gross invasion of the blood stream occurs at times and is followed by small hemorrhages in the skin and darkening of the latter, this symptom being responsible for the name Black Death given to some of the epidemics in the past. As considered earlier, the plague bacillus can give rise to a pneumonia, pneumonic plague spreading readily from man to man. The bubonic type does not spread as readily from man to man and is usually spread by infected fleas, the great epidemics of history always being



associated with war, famine, and crowding, which means closer contact with rats and their vermin.

*Pasteurella pestis* is a short, plump, polar-staining, gram-negative rod, 0.5 to 0.7 by 1.5 to 1.7  $\mu$  in size. It is encapsulated in the animal body and in young cultures. The cells are extremely pleomorphic in old cultures. No exotoxin is produced, but a toxic cellular component or endotoxin is liberated upon autolysis of the cells. *P. pestis* will grow on plain agar and in milk, characteristics which serve to differentiate it from the more exacting *P. tularensis* of tularemia.

Immune sera have relatively little value against plague, even though an individual who has recovered from the disease is permanently immune. This suggests a tissue rather than a general type of immunity. Sulfadiazine and streptomycin or other antibiotics are effective against the organism. Immunization can be induced following the injection of heat-killed organisms or other types of vaccine, but the most effective control measure is control of the rat population. In endemic areas the spread of *P. pestis* can be prevented by effective spraying with DDT, this agent being toxic to the rat flea.

**Typhus and Rocky Mountain Spotted Fevers.** These diseases are caused by rickettsiae, organisms we have considered as being intermediate in their characteristics between the bacteria and the viruses. Typhus fever is caused by *Rickettsia prowazeki*, Rocky Mountain spotted fever by *R. rickettsi*, and the closely related diseases known as scrub typhus, tsutsugamushi, and tropical typhus by *R. tsutsugamushi*.

Typhus fever exists in two forms, epidemic and endemic (murine). The former is spread from man to man by means of the body louse, the latter from rat to rat or from rat to man by means of the rat flea. The endemic, or murine type, apparently is caused by a rickettsia other than *R. prowazeki*, the name *R. typhi* (also *R. mooseri*) being proposed for it. The epidemic form of the disease is under control in most areas at the present time, and its spread, when the disease does occur, can best be prevented by use of DDT to control the louse population of man. Like plague, it is an infection associated with war, famine, crowding, and filth. Vaccines, prepared from rickettsiae grown in the developing egg, appear to be of value for immunization of individuals apt to be exposed to the infection.

Endemic typhus is of more concern in the United States than epidemic typhus, the former disease being prevalent in Mexico and the southern parts of this country. The rat serves as a reservoir of the infection. Clinically it is similar to epidemic typhus but is generally less severe. An oddity associated with both infections is that infected persons develop antibodies that will agglutinate certain strains of the bacterium *Proteus*

*vulgaris*, this organism not being associated with the infections but apparently having an antigen similar in reactivity to an antigen also present in the rickettsiae. Chloramphenicol and the tetracyclines are effective, while the sulfa drugs may stimulate the infection.

Rocky Mountain spotted fever rickettsiae are hereditarily transmitted (in the eggs but not by genes) from tick to tick, this insect being the natural host for *R. rickettsi* and transmitting it to man. The disease is similar to endemic typhus fever and occurs sporadically throughout much of the United States, the main areas of infection being parts of Idaho and Montana. Similar tick-borne infections are present in various parts of the world and are apparently due to strains of this organism. Vaccines can be prepared for the immunization of individuals apt to be exposed to ticks in areas where the infection is present.

**Yellow Fever.** Yellow fever is caused by one of the smaller filtrable viruses, 19 to 22  $m\mu$  in diameter. It is a disease prevalent in tropical and subtropical countries and is transmitted to man by the bite of an infected mosquito but not from man to man directly. Certain jungle monkeys may serve as a reservoir of the infectious agent. Yellow fever, together with malaria, made large parts of the West Indies and Central and South America dangerous for the white man in the last century, and the disease did spread to the United States, one devastating epidemic occurring in New Orleans in 1878.

An understanding of this disease developed from the dramatic studies of Reed, Carroll, and others in Cuba during the Spanish-American War. They proved, with the aid of soldier volunteers, that the disease is apparently of viral origin, that it is transmitted solely by the bite of infected mosquitoes (*Aedes atgypti*), and that a mosquito, if it is to become infective, must bite the patient within 3 days of the onset of the disease, the mosquito becoming infective for man after about 12 days. Control of the mosquito populations and prevention of contact of mosquitoes with cases of yellow fever made possible the construction of the Panama Canal.

The virus of yellow fever is viscerotropic; i.e., it tends to localize in the viscera. Theiler, in 1930, passed the virus intracerebrally in series in mice and noted that it tended to lose its viscerotropic characteristics, becoming adapted to multiplication in nervous tissues. The neurotropic variant was not infective for man by the subcutaneous route of injection but retained its antigenic identity. The neurotropic strain was later adapted for growth in the developing egg, both neurotropism and viscerotropism being depressed without loss of immunizing properties. Vaccines prepared from this variant are available and quite effective. This serves as an example that variation does occur in the smaller viruses as well as in the larger ones such as rabies (demonstrated by Pasteur) and in the

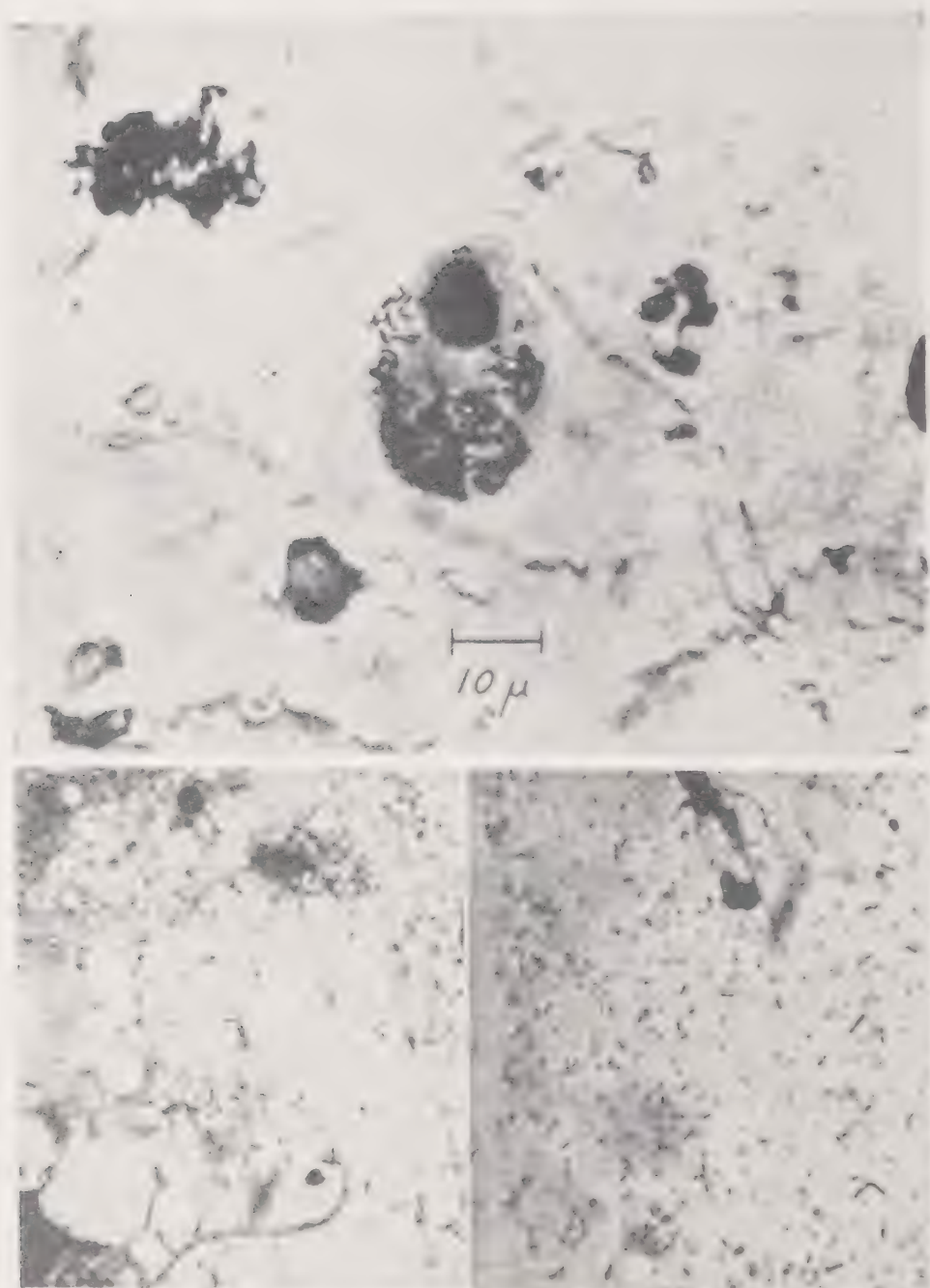


FIG. 23-10. Photomicrographs of the rickettsiae of endemic (1 and 3) and epidemic (2) typhus stained with Masson-Goldmann's stain. [From Blaz, Smadel, Anderson, and Chambers, *Journal of Experimental Medicine*, 77, 355 (1943).]



bacteria or other true microorganisms. Control of *Aedes aegypti* remains as the best over-all method of prevention of yellow fever.

**Malaria.** Malaria, an infection of protozoan origin, is one of the most common and widespread diseases of man. It is most frequent in tropical and subtropical areas, being responsible for an estimated 3 million deaths per year with possibly 100 times as many actual cases of the disease. The malarial protozoa belong to the class Sporozoa and the genus *Plasmodium*. Four distinct species are recognized, *P. vivax*, *P. falciparum*, *P. malariae*, and *P. ovale*, each being responsible for specific forms of the diseases collectively known as malaria. These organisms pass through a distinctive life cycle, a sexual cycle in the body of the female mosquito and an asexual one in man. Asexual multiplication occurs within the blood cells of man, the time required for multiplication varying with the species. After asexual multiplication has gone on for a number of days, sexual forms begin to develop. These sexual forms are infective for the mosquito and are obtained by them in blood serving as a meal. Male and female elements must be obtained from man, the male forms fertilizing the female ones when both are present in the mosquito. The fertilized cells undergo a number of changes with the final formation of sporozoites, spore-like bodies which are infective for man. The complete cycle is quite complex and is described in detail in the specialized texts. The life cycles of the protozoan parasites and of the higher fungi suggest that life cycles may be involved in the history of the lower pathogenic parasites, but information on this point at the present time is only suggestive.

Malaria can be best controlled by control of the mosquito population, but this is an even more difficult task than with yellow fever, where only one species is involved. Drainage of swampy areas and oiling of breeding waters is of considerable value, but the use of DDT and other antiinsect sprays on a large scale is more promising. No method of immunization is available, but chemotherapy with quinine is one of the oldest specific treatments known to man. Antimalarial drugs such as atabrine have been developed within recent years.

**Miscellaneous.** No attempt has been made to cover all the agents pathogenic for man. Particular attention has been placed on the pathogenic bacteria. A number of species were not mentioned, and many others exist that are pathogenic for man, animals, and plants. The same statement holds true for the protozoa and even higher forms of animal life, particularly the worms. In most cases the pathogenic forms are parasitic ones. This leads to an essential similarity in all infectious diseases, a struggle for existence between a parasite and its unwilling host. The nature of the struggle is dependent upon the properties both of the parasite and of its particular host and these properties are subject to change



in the variations normal to the host and to the parasite. A proper understanding of infectious diseases requires thorough knowledge of both parasite and host and of the interrelationships between the two. Much is known; much remains to be learned, but progress is being made, and at some future date it may be possible to summarize the more important infectious diseases and their causative agents in a more specific manner than was possible in the foregoing discussion.

## CHAPTER 24

### INFECTIOUS DISEASES OF ANIMALS AND PLANTS

The general principles of infectious diseases in animals and plants are the same as those for infectious processes in man, but the details do vary in that different hosts and in most cases different parasites are involved; hence there will be differences in the host-parasite relationships. In addition, there is little evidence indicating that antibodies or their equivalents develop during the course of plant diseases. We can only summarize here the more general nature of the infectious agents responsible for the more common animal and plant diseases. Many of these diseases are of economic importance in that they threaten our sources of food, drink, clothing, and building materials. It has been estimated that diseases of farm animals and poultry are responsible for a loss of around one billion dollars annually in the United States, this figure being based both on loss of animals and from decreased productivity. The loss from diseases of food and other crops would also yield a staggering figure, possibly around three billion dollars.

#### ANIMAL DISEASES

Infectious diseases of animals are of importance not only economically; domesticated and wild animals and birds serve in many instances as reservoirs of agents pathogenic for man as well as for other animals. Selected examples of such infectious agents—anthrax, brucellosis (undulant fever or contagious abortion), bovine tuberculosis, glanders, tularemia, bubonic plague, streptococcal infections, hemorrhagic jaundice, rat-fate fever, endemic typhus, psittacosis, cowpox, and rabies—were considered in the preceding chapter. These diseases and their causative agents will not be considered further except where desirable to illustrate the discussion. It might be well to point out that animals are not only subject to natural infections but that many species have served us well in the laboratory study of agents infectious only for man under natural conditions.

**Bacterial Diseases of Animals.** Tuberculosis formerly was one of the major diseases of cattle and swine, the progressive disease in cattle being

caused only by the bovine strain of *Mycobacterium tuberculosis*. Legislation accompanied by active field work since 1917 has practically eradicated this source of human infection in the United States and in many other countries as well, pasteurization of milk serving as a further safeguard. Animals found to be tuberculin-positive reactors were eradicated, thus interrupting the chain of infection of other animals and of man. Naturally drastic measures of this type cannot be employed in the control of human infections, but they can be applied where the immediate economic loss is offset by long-term gains.

Many swine are infected but to a great extent the infection is induced by the avian strain (*M. avium*) of the tubercle bacillus and the lesions tend to remain localized in the neck. This infection is not particularly debilitating to swine and is not important from a public-health point of view. Chickens commonly serve as a reservoir for *M. avium*, and the incidence of this type of tuberculosis in hogs is much greater in those from small farms where they come into close contact with chickens. Hogs are of greater danger to man as a carrier of the worm *Trichinella spiralis*, the causative agent of trichinosis. This worm is parasitic in rats, spreads to hogs which eat infected carcasses (or uncooked garbage), and then reaches man through improperly cooked pork. Personal protection can be assured by thorough cooking of pork or pork products or storage of uncooked pork for 24 hours at  $-18^{\circ}\text{C}$ .

We have seen that cattle, swine, and goats are subject to contagious abortion induced by the *Brucella*. Eradication of cattle whose serum (Bang's test) or milk shows an antibody titer greater than normal in agglutination tests has greatly reduced the incidence of this disease. It has been further reduced as the result of immunization of calves with a living vaccine prepared from a *Brucella* of low virulence, calves being more resistant than adult animals. Sanitary procedures, proper disposal of contaminated material, and the use of *Brucella*-free breeding stock serve as further control measures.

Cattle are also subject to mastitis, an infection inducing inflammation of the udder and consequent reduction in quantity and quality of the milk produced. The chronic type of this infection commonly is caused by staphylococci or *Streptococcus agalactiae*, while a number of bacterial species are responsible for the more acute type of infection. *Streptococcus pyogenes* is a causative agent of the acute type and can be transmitted from infected animals to man through milk. Man can also infect cattle. Efficient sanitary procedures and cleanliness in the dairy serve to prevent the spread of the infection in a herd. Proper use of antibiotics to which the causative agent is susceptible is also of value but will not necessarily completely control the infectious agent in vivo.

Anthrax is of minor importance as an infectious disease of animals

in the United States but is of greater incidence in European pastures, which are more heavily infested with the spores of *Bacillus anthracis*. Its spread can be controlled to a great extent by prompt disposal of infected carcasses and by immunization procedures. Anthrax-like diseases are observed in various animals infected with organisms such as *Clostridium septicum* and *C. fesiari tchaurœi*. The latter anaerobe causes a disease known as "quarter-evil," or blackleg, chiefly in sheep, goats, and cattle, but particularly in cattle. Infection results primarily from the entrance of contaminated soil into wounds on the lower extremities, many of the symptoms of the disease being induced by exotoxins formed *in vivo*. Immunization procedures are available to protect animals against the agent where it is prevalent. *C. septicum* is pathogenic for man as well as for animals, while *C. fesiari* is not.

Vibriosis has long been recognized as a disease of animals and can be caused by a number of vibrios. *Vibrio fetus* appears to be the major pathogen of this type in the United States, but the high incidence of this infection was not recognized before 1948. Infection with *V. fetus* decreases fertility and induces early abortion in cattle and sheep. Prevention of this disease depends primarily upon the detection and destruction of infected animals, thus interrupting the spread of the bacterium.

Leptospirosis is another recently recognized (about 1940) major disease of cattle and hogs in the United States. It is caused by the spirochete *Leptospira pomona* and, like Weil's disease in man (*Leptospira icterohemorrhagiae*), is spread primarily by food or water contaminated with urine from infected animals. *L. pomona* was not recognized as a distinct species in the sixth edition of Bergey's Manual, and some confusion exists concerning it. This *Leptospira* may also be of limited pathogenicity for man, diseases known as swamp fever and swineherd's disease supposedly being caused by it. This infection in animals is characterized by failure of the animals to gain weight properly, decreased milk production, abortion at times, and frequently death of the infected animals. Typical symptoms and serological tests can be employed for diagnostic purposes. The spread of the infection can be hindered most readily by sanitary procedures, primarily by prevention of contamination of the herd's supply of food and water by urine from infected animals.

**Viral Diseases.** A considerable number of infectious diseases of wild and domesticated animals are caused by filtrable viruses, some of which we have seen are also pathogenic for man. Epizootics (epidemics among animals) of hog cholera have been responsible for major losses, and still occur, but to a lesser extent. Hog cholera is caused by a small virus (about 35 *mμ*) which is spread by secretions and excretions from infected animals via contaminated food or water to healthy swine. Immunization



procedures are of value in prevention of the disease, the early practice being to inject separately blood or tissue suspensions containing the virus from infected animals and serum from hyperimmunized ones. The neutralizing antibodies in the serum inhibit the virus in the blood or tissue preparations from infected animals, but the virus may remain active or multiply for a time in the vaccinated animal. At least the production of sufficient antibody to protect the vaccinated animal is elicited as a result of the procedure. This procedure is not without danger, since the activity of the virus or immune serum may vary. Vaccines containing virus attenuated in virulence for the hog, by passage in rabbits, appear to be safer and of value for immunization.

Another viral disease of hogs—swine influenza—is of considerable economic importance and at the same time of biological interest. This disease is caused by the combined activity of two organisms, *Hemophilus influenzae suis* and a virus antigenically related to type A influenza virus. Speculation exists concerning possible relationship of the hog influenza virus to the 1917 pandemic of influenza in man. Shope has shown that a rather complex cycle for the transmission of the hog influenza virus exists in nature. Lungworms (nematodes) in infected animals become infected, and the virus is excreted in the hog's feces in ova from the worms. The virus appears to be in an inactive or "masked" form in the ova. The lungworm ova are ingested by earthworms, in which the lungworm larvae then develop. The virus enters hogs which eat earthworms containing lungworm larvae carrying the masked virus. Contamination of healthy hogs in this manner does not suffice to induce the infection, but an "exciting" factor or stimulus is involved. Injection of *H. influenzae suis* into the contaminated animal can act as an exciting cause, but climatic conditions are also involved, outbreaks generally occurring during the colder months of the year. It is possible that a number of the viruses may exist in masked form in nature and their spread to susceptible hosts may be dependent upon cycles such as the above. Successful immunization procedures for the prevention of hog influenza have not been developed.

Foot-and-mouth disease is one of the most contagious infectious diseases, particularly in cattle. It is caused by one of the smallest viruses (about 10  $m\mu$ ). The disease is characterized by vesicular eruptions in the mouth, around the hoofs, and on the udders of female cattle. The infection is debilitating but not generally fatal unless complicated by secondary invaders. The virus is spread via milk from infected animals and by material from the vesicular eruptions, chiefly through the agency of contaminated food or water. Cattle handlers can also spread the virus by means of their hands or clothing contaminated with it, but man is not particularly susceptible. The major control of spread of the disease

consists of quarantining infected herds and killing the diseased animals. The bodies should be treated with chloride of lime and buried. Vaccination with virus attenuated by growth in tissue cultures is of value as a prophylactic measure. Strict quarantine on animals or meat from countries in which the disease is prevalent is enforced in the United States. Vesicular exanthema of swine and vesicular stomatitis are closely related viral diseases of animals.

Newcastle disease is a viral disease of birds that is highly infectious and the spread of which, like that of foot-and-mouth disease, is controlled most readily by quarantine and destruction of infected poultry. An egg-grown attenuated virus is of value as an immunizing agent, being applied in the form of a dry powder either intranasally or to the conjunctiva of young chicks. Fowl plague and fowl pox are other major virus diseases of poultry.

Canine distemper is caused by a virus which is quite infective for dogs, particularly young ones, and which may cause serious losses in mink and fox farms. Immunization with appropriate vaccines is an effective measure to employ on our pets. Other virus diseases of dogs and cats include rabies, canine hepatitis, and cat enteritis (cat distemper).

Insects, like the higher forms of life, are subject to attack by microorganisms and viruses. The insect viruses are of economic importance in the silk industry, in honey-bee broods, and in particular in the natural control of the insect populations. Attempts have been made to control epidemics of insects destructive to plants of economic importance to us by artificial inoculation of insects with a virus to which they are susceptible. For example, alfalfa fields have been sprayed with material containing a virus causing a polyhedrosis type of disease in the alfalfa caterpillar. Such measures are somewhat effective under favorable conditions in reducing the insect population, but it is difficult to start an artificial epidemic in nature. This indicates the difficulties that could be inherent in biological warfare, particularly since man could initiate countermeasures not available to the insect.

A group of viruses collectively known as the encephalitides is commonly associated with or transmitted by insects to man and other animals. These viruses are neurotropic in character, i.e., tend to multiply in neural tissue and to induce inflammation of the brain and spinal cord. They are relatively small (25-50  $m\mu$ ) and closely related to each other, being distinguished primarily on the basis of serological reactions. There appears to be quite definite geographic distributions of these agents, evidenced by names such as western and eastern equine encephalomyelitis or Saint Louis, California, Japanese B, and Murray Valley encephalitis. Equine encephalomyelitis is a disease of horses that may appear

in epizootic form and is characterized by symptoms ranging from lassitude to excitability and wildness, a fatality rate near 20 per cent being observed in untreated animals. Man may acquire the disease from horses. The mosquito has been incriminated as a vector for the virus and there is considerable evidence that the virus is maintained in nature in birds, possibly a mosquito-bird-mosquito cycle operating during the "mosquito" months and a mite-bird-mite one at other times. Ticks and other insects may serve as vectors for this or other viruses of the encephalitis group, and chickens or other birds as natural reservoirs of the viruses. It is of interest that around 1936 it was observed that formalinized preparations of nervous tissue from infected animals served as a protective vaccine for horses. Encephalitis virus was obtained in higher concentration later by growing it in the embryonated egg, and formalinized vaccines prepared therefrom were more effective than the tissue ones. These observations showed that some formalin-treated, artificially cultured viruses would serve as effective vaccines and paved the way for the later development of the poliomyelitis vaccine for man.

A number of tumors in chickens (fowl sarcoma and avian leucosis), papillomas in many animals, and mouse mammary cancer have been shown to be caused by viruses. This raises the question of a possible viral origin of tumors and cancers in man. Some suggestive evidence has been obtained, but the concept has not been definitely established.

### INFECTIOUS DISEASES OF PLANTS

Plants, like animals, have their parasites, and some of these parasites are destructive to the tissues of the plant. Certain fungi were recognized as plant pathogens around the middle of the nineteenth century, but the recognition of bacteria as plant pathogens was not general until early in this century, despite the reports of Burrill (1882) on the bacterial origin of fire blight of apple and pear trees and of Wakker (1883) on yellows of the hyacinth. This skepticism was not overcome until about 1900, the studies of Theobald Smith, one of America's leading bacteriologists, definitely establishing the bacterial origin of a number of plant diseases. As has already been mentioned, the studies of Iwanowski and of Beijerinck established a filtrable agent as the cause of tobacco mosaic, this concept apparently being easier to establish than that of bacteria as plant pathogens. At the present time over 200 species or varieties of bacteria have been recognized as plant pathogens. All are asporogenous, gram-negative (with nine exceptions) rods with relatively simple metabolic activities and nutritional requirements. It is odd that apparently none attack cellulose and relatively few hydrolyze starch, two of the major constituents of plants. In general, the plant pathogens are more closely



related to the soil bacteria than to the animal pathogens and tend toward saprophytism rather than parasitism.

Identification and classification of the bacteria pathogenic for plants are extremely difficult in many instances, and much confusion still exists. For example, *Xanthomonas corylina* and *X. juglandis* appear identical in the laboratory; yet the former is pathogenic on filberts, the latter on walnuts, and they will not cross-infect. Most plant pathogens, with the exception of the genus *Erwinia* in the family Enterobacteriaceae, were formerly placed in a single genus *Phytomonas*. Now they are scattered in several genera. Approximately one-half of the plant pathogens produce green fluorescent pigments and are placed in the genus *Pseudomonas*. The genus *Xanthomonas* includes the next largest group, polarly flagellated rods which produce yellow pigments. A number of species which produce abnormal growths on roots and stems are placed in the *Agrobacterium*; the gram-positive species are in the genus *Corynebacterium*, and a limited number of miscellaneous forms are in the genus *Bacterium*.

**Types of Plant Diseases.** The diseases of plants vary in their symptomatology in a manner analogous to animal diseases, and in many respects the nature of the disease depends upon similar properties in both cases, portal of entry, invasive power, mode of spread, and toxicity. Antibody response on the part of the host, however, appears to be lacking, and the plant in general appears to have less specific means of resistance to the invader once the external barriers have been crossed. Natural immunity, the resistance of races or species, or of strains in a species, is better known than in animals, breeding experiments having developed many strains or types resistant to specific bacteria or higher fungi such as the rusts.

*Leaf and fruit spots* are localized infections, somewhat analogous to localized abscesses in man, while the more generalized infections are known as blights. Species of *Pseudomonas* and *Xanthomonas* are frequently responsible for the former, other species of the same genera and

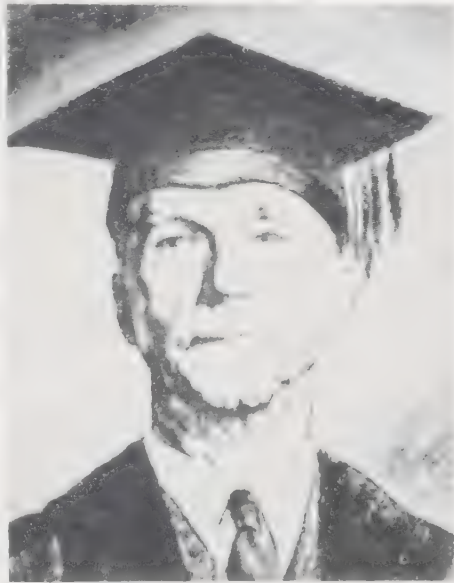


FIG. 24-1. Theobald Smith, one of America's early leading bacteriologists. (From a portrait by Montgomery, courtesy of the Central Scientific Co., Chicago.)



also of *Erwinia* (e.g., *E. amylovora*, pear and apple blight) for the latter type of infection. In some infections the organisms multiply and accumulate in large numbers in the vascular system, frequently interrupting the flow of sap, and the plant droops or wilts. *Corynebacteria* are frequently responsible for the *vascular diseases*, or *wilts*, although other genera, particularly *Pseudomonas* and *Bacterium*, may contain species eliciting wilts. A limited number of plant diseases are in part caused by bacterial toxins, the wildfire of tobacco involving toxin production by *Pseudomonas tabaci*. Another group of plant diseases is characterized by the formation of abnormal growth, *tumors* or *galls*. The cells in a local area appear to be stimulated to abnormal multiplication with little or no tissue destruction. Actually nodule formation induced by rhizobium on the roots of leguminous plants could be considered an example of this type of infection. *Agrobacterium tumefaciens* is a common example of this type of plant pathogen. It produces crown gall on a variety of plants. Finally, there is a group of diseases characterized by, and called *soft rots*. Species of *Erwinia* are generally involved and rapidly spread through the tissues with the aid of the enzyme pectinase, which hydrolyzes pectin, a cementing material that binds the plant cells together. The tissues are not only killed; they are reduced to a soft, moist, pulpy mass. The soft rot of carrots is a typical example, *E. carotovora* being the causative agent.

Plants, as a general rule, are more subject to infections induced by the higher fungi than are animals. Actually the majority of infectious diseases of plants are caused by the multicellular fungi we commonly speak of as molds. Blights, scabs, smuts, rusts, and wilts of various sorts often are of fungal origin. The home gardener commonly loses many of the seedlings he so fondly nurses because of damping-off induced by fungi. These fungi may attack the plant before the shoot pushes through the ground, or their destructive nature may become apparent just as the young seedling appears to be thriving. Damping-off can be controlled by disinfection of the seed and the use of fungicides or, on a small scale, by disinfection of the soil. The problem becomes more serious on a large scale for the commercial gardener and the farmer.

Two classical diseases of fungal origin that have markedly influenced the welfare of man are late blight of potatoes and stem rust of wheat. In 1845 almost the entire potato crop in Ireland was wiped out following a rainy, muggy July which provided ideal conditions for the growth of the causative agent, *Phytophthora infestans*, on the potato vines. The leaves are destroyed, necrotic areas forming on the edges of the leaf and spreading inward. The mold growth appears downy and yellowish (silver) in color. Spores are formed in large numbers and are transmitted by birds, insects, man, and wind to nearby healthy plants, thus spreading the re-



FIG. 24-2 Crown gall on a tomato plant.

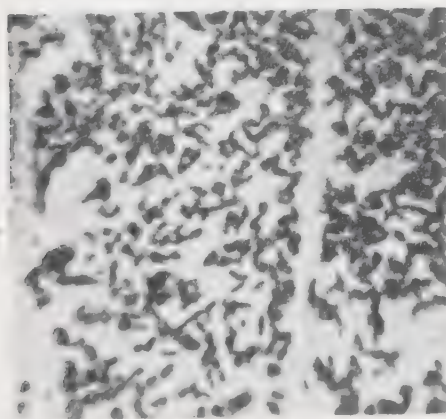


FIG. 24-3 *Agrobacterium (Phytophthora) tumefaciens*, the bacterium producing the crown gall illustrated in Fig. 24-2.

fectious agent. If climatic conditions are just right, the blight spreads and progresses rapidly. Since potatoes formed the bulk of the Irish diet, famine occurred as a result of the blight. It has been estimated that this plant epidemic was the cause of around 500,000 deaths from starvation and was responsible for the emigration of even more people to other countries. Shortly thereafter, Berkeley in England suggested that the blight was caused by a fungus, and this was established by DeBary in 1855, a number of years before microbial causes of infectious diseases in man and animals were discovered.

Potato blight also attacks the tubers in storage and causes them to rot if they are not stored at a low temperature under dry conditions. Spores may be carried over the winter on seed potatoes, and the fungus can attack the planted tubers and roots, spreading from there to the stems and foliage. Local epidemics start more readily, however, from spoiled and cull potatoes that are thrown away and allowed to rot. Large numbers of spores are produced and spread via the air to potato patches. One of the most important control measures is to bury or burn all spoiled or discarded potatoes. Spraying the potato plants with a fungicide is of value in preventing the spread of the blight once it appears in a potato field. The fungus also causes a blight of tomatoes, an epidemic in the United States in 1946 causing an estimated loss of forty million dollars.

Stem rust of wheat, other grains, and grasses has also been the cause of famine in many parts of the world. This disease is caused by *Puccinia graminis*, a fungus with a highly complex life cycle. There are a number of varieties of this organism and many different strains or races. It attacks, for example, the young leaves and stems of wheat, rusty red spots appearing on them at first and gradually turning black. The fungus utilizes materials in the plant that in part, at least, would have been used in the formation of the grain (seeds), and as a result the yield is decreased and the kernels are of poor quality. In the northern parts of the United States the barberry serves as a host for this fungus, and part of the fungus's life cycle is completed on this plant. Eradication of the barberry serves as one control measure. Another control is the development of rust-resistant plants, but new strains of the fungus do arise upon the originally resistant plants. The use of sulfur dusts and good agricultural practices are of further aid in the control of stem rust. Weather is another important factor in that moist, warm conditions favor growth of this and many other fungi, but it is a factor beyond our control.

Fungi attack not only living plants but also our foods obtained from them. This was illustrated above in the case of potato rot. Spoilage of grains and dried grasses (hay) stored for future use is induced by fungi and this is responsible for huge losses annually.

A large number of plant diseases are of virus origin, it being said that



almost all cultivated and many wild plants are subject to infection by at least one virus. Some of the more important groups of virus diseases of plants are the *mosaic*, the  *yellows*, the *ring-spot*, the *spotted-wilt*, the *leaf-scumming* (crinkling), and the *leaf-curl* groups, the names indicating the general symptoms observed. It is of interest that the first virus to be highly purified and crystallized was the virus of tobacco mosaic, which was also the first virus to be connected with a disease. The literature on the fungus, bacterial, and virus diseases of plants is almost as voluminous as that on the infectious diseases of man, and advances in one field have frequently contributed to advances in the other.

**Control of Diseases of Plants.** In many instances fungus diseases of plants can be controlled to a considerable extent by the use of appropriate sprays, copper solutions frequently being employed. Sprays in general have little value against bacterial diseases of plants. One of the best methods of control is complete destruction of all infected plants or parts of plants. Care must be exercised, for example, in pruning diseased parts from trees to see that the pruning implements do not become infected and transmit the bacteria to healthy parts of the tree. In some instances the spread of pathogens can be greatly reduced by disinfection of the seeds, black rot of cabbage, caused by *X. campestris*, being greatly reduced when the seeds are heated to 122°F. Once land becomes infected, it is generally advisable to shift to other crops, since disinfection of soil on a large scale is impracticable. Crop rotation, proper cultivation and fertilization, careful disposal of wastes, and the use of resistant strains or seed from vigorous races of plants are practices that aid markedly in the control of plant diseases. It might be mentioned that balances tend to be established in nature, and when we interfere with these balances, shifts sometimes occur that are detrimental to us. For example, orange groves were sprayed with a fungicide to control a fungal disease of the leaves and fruit. This was successful, but it also killed a fungus parasitic upon scale insects. These insects multiplied to a much greater extent than normal and did considerable damage to the orchards.

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## APPENDIX

Condensed description of the organisms classified as Eubacteriineae in the sixth edition of "Bergey's Manual of Determinative Bacteriology" (The Williams & Wilkins Company, Baltimore, 1948).

The classification of bacteria is in a state of flux. The classification and nomenclature employed in this text is that presented in the sixth edition of Bergey's Manual. The following description and classification of the members of the Eubacteriineae illustrates the principles involved, and is presented for reference purposes (see Chap. 6 for further discussion and for details of the proposed reclassification).

**Nitrobacteriaceae.** These are the bacteria capable of growing in an inorganic medium with carbon dioxide as the sole source of carbon. They obtain their energy from the oxidation of inorganic compounds, the energy source being quite specific for each species or genus. With minor exceptions they are gram-negative rods found in the soil and in water. These bacteria are of extreme importance in the economy of nature, many of them converting inorganic matter into compounds more readily utilized by plant life in general. The Nitrobacteriaceae is subdivided as follows:

<i>Tribe</i>	<i>Genus</i>	<i>Major characteristics</i>
Nitrobacterieae .....		Oxidize ammonia to nitrite
	<i>Nitrosomonas</i> .....	Ellipsoidal cells
	<i>Nitrosococcus</i> .....	Spherical cells
	<i>Nitrospira</i> .....	Spiral cells
	<i>Nitrosocystis</i> .....	Cell masses surrounded by a common membrane
	<i>Nitrosogloea</i> .....	Cells embedded in slime
		Oxidize nitrite to nitrate
	<i>Nitrobacter</i> .....	Free rods
Hydrogenomonadeae	<i>Nitrocystis</i> .....	Cells embedded in slime
Thiobacilleae	<i>Hydrogenomonas</i> ..	Oxidize hydrogen gas
	<i>Thiobacillus</i> .....	Oxidize sulfur, thiosulfate, sulfide, or similar sulfur compounds

**Pseudomonadaceae.** This family consists of elongate, gram-negative rods (either straight or curved) usually motile by means of polar flagella. For the most part they grow well and rapidly on simple organic media, preferably under aerobic conditions. The family contains colorless and

pigmented species, most of which are saprophytes, but a few are parasitic upon plants or animals. The Pseudomonadaceae is divided as follows:

Tribe	Genus	Major characteristics
Pseudomonadeae .....		Straight rods
	<i>Pseudomonas</i> .....	Bluish- or yellowish-green, water-soluble pigment
	<i>Xanthomonas</i> .....	Yellow, water-insoluble pigment. Mostly plant pathogens
	<i>Methanomonas</i> .....	Oxidize methane
	<i>Acetobacter</i> .....	Oxidize ethyl alcohol to acetic acid
	<i>Protaminobacter</i> .....	Oxidize protamines
	<i>Mycoplana</i> .....	Branching cells. Oxidize benzene derivatives such as phenol
Spirilleae .....		More or less curved cells, vibrio type has single, polar flagellum
	<i>Vibrio</i> .....	Short, slightly curved rods
	<i>Desulfovibrio</i> .....	Slightly curved rods. Strict anaerobes, reducing sulfate to sulfide
	<i>Cellvibrio</i> .....	Long, slightly curved rods with rounded ends. Oxidize cellulose
	<i>Cellfalcicula</i> .....	Short, curved rods with pointed ends. Oxidize cellulose
	<i>Thiospira</i> .....	Spirilla oxidizing inorganic sulfur compounds. Intracellular sulfur granules
	<i>Spirillum</i> .....	Spirilla other than <i>Thiospira</i>

**Azotobacteriaceae.** This family is composed of relatively large rods or cocci, often yeast-like in shape, which are highly pleomorphic. The cells are obligate aerobes primarily characterized by their ability to utilize nitrogen gas as the sole source of nitrogen, nitrogen fixation occurring independently of other forms of life. There is but one genus, *Azotobacter*.

**Rhizobiaceae.** This family is composed of sparsely flagellated, gram-negative rods which utilize sugars with little or no acid formation. It is a rather heterogenous family in that one genus is characterized by its ability to fix nitrogen in symbiosis with legumes, another by the production of violet pigment. A number of the species are pathogenic upon plants, giving rise to the formation of abnormal growths. The family Rhizobiaceae is divided as follows:

Genus	Major characteristics
<i>Rhizobium</i> .....	Cells capable of fixing free nitrogen symbiotically with legumes
<i>Agrobacterium</i> .....	Plant pathogens or saprophytes, nonpigmented
<i>Crocmobacterium</i> .....	Primarily saprophytes which form a violet pigment

**Micrococcaceae.** This family is composed of heterotrophic, generally gram-positive cocci which show little or no tendency to form chains.

At least one species forms endospores, and a few species are motile. There are both saprophytic and pathogenic species in the family Micrococcaceae, which is divided as follows:

Genus	Major characteristics
<i>Micrococcus</i> .....	Cells generally in irregular groups and frequently pigmented, yellow to red
<i>Gaffkya</i> .....	Tetrad arrangement of cells, white to yellow pigment
<i>Sarcina</i> .....	Cubical or regular packet arrangement of cells, often yellow to orange pigment

**Neisseriaceae.** This family is composed of gram-negative, parasitic cocci which occur in pairs or masses. These bacteria are rather exacting in their nutritional requirements, growth generally being sparse on ordinary media. The family Neisseriaceae is divided into two genera as follows:

Genus	Major characteristics
<i>Neisseria</i> .....	Cells tend to occur in pairs with adjacent sides flattened. Limited biochemical activity
<i>Vibrio</i> .....	Smaller cells but more active biochemically than the <i>Neisseria</i> . Generally occur in masses

**Lactobacteriaceae.** This family is composed of both gram-positive rods and chain-forming cocci which tend to grow most readily under anaerobic conditions, fermenting sugars with the production of considerable quantities of lactic acid. It contains both saprophytic and parasitic species which are rather widely distributed in nature. The Lactobacteriaceae is divided into two tribes and seven genera as follows:

Tribe	Genus	Major characteristics
Streptococceae.....		Chain-forming, highly fermentative cocci
	<i>Diplococcus</i> .....	Cells usually in pairs, aerobic species soluble in bile
	<i>Streptococcus</i> .....	Cells usually in chains, not soluble in bile
	<i>Leuconostoc</i> .....	Spherical to pointed cells differing from above genera in that both acid and gas are produced during fermentation
Lactobacilleae.....		Nonmotile rods, often long and slender
	<i>Lactobacillus</i> .....	Lactic acid always produced, catalase absent
	<i>Microbacterium</i> .....	Catalase positive, lactic-acid formers
	<i>Propionibacterium</i> ..	Fermentation products are propionic and acetic acids, and carbon dioxide
	<i>Butyribacterium</i> ....	Fermentation products are butyric and acetic acids, and carbon dioxide

**Corynebacteriaceae.** This family is composed of gram-positive, frequently banded or beaded rods showing considerable diversity of form. Most species are parasites on animals, a few being parasitic on



plants. Many of these organisms were formerly classified with the mycobacteria under the Actinomycetales rather than as at present in the Eubacteriales. Three genera are recognized as follows:

<i>Genus</i>	<i>Major characteristics</i>
<i>Corynebacterium</i>	Rods which are variable in form and staining properties
<i>Listeria</i>	Flagellated rods causing a monocytosis in warm-blooded animals
<i>Erysipelothrix</i>	Cells which tend to be filamentous and are pathogenic in warm-blooded animals

**Achromobacteriaceae.** This family is composed of gram-negative rods which are usually uniform in shape. They differ from the following family, the Enterobacteriaceae, in that they are much less active metabolically, fermenting carbohydrates feebly or not at all. They are for the most part saprophytic forms. Three genera are recognized as follows:

<i>Genus</i>	<i>Major characteristics</i>
<i>Alcaligenes</i>	Does not ferment carbohydrates
<i>Achromobacter</i>	Nonchromogenic cells which usually ferment glucose with acid production
<i>Flavobacterium</i>	Yellow to orange pigmented species which usually ferment glucose with acid production

**Enterobacteriaceae.** This is one of the largest families of the Eubacteriineae and is composed of gram-negative rods which ferment glucose with the production of acid or of acid and gas. Most species grow readily on simple culture media. They tend to be parasitic upon plants or animals or to be associated with decomposing plant materials. Five tribes and eight genera are recognized as follows:

<i>Tribe</i>	<i>Genus</i>	<i>Major characteristics</i>
Eschericheae		Ferment glucose and lactose with the formation of acid and gas
	<i>Escherichia</i>	Produce considerable amounts of acids from glucose but no acetylmethylcarbinol
	<i>Aerobacter</i>	Produces acetylmethylcarbinol, acid production not as great as above
	<i>Klebsiella</i>	Biochemically more variable than above genera
Erwineae		Primarily plant pathogens
	<i>Erwinia</i>	Somewhat more dependent on preformed organic nitrogenous matter than the Eschericheae
Serrateae		Saprophytic, red pigmented rods
	<i>Serratia</i>	Metabolically similar to Eschericheae
Proteae		Ferment glucose but not lactose with the formation of acid and usually gas, and urea is decomposed
	<i>Proteus</i>	Colonies tend to spread on moist agar

Tribe	Genus	Major characteristics
Salmonelleae .....		Lactose rarely fermented and urea not decomposed
	<i>Salmonella</i> ....	Most species are motile, and the majority ferment glucose with acid and gas production
	<i>Shigella</i> .....	Nonmotile and ferment glucose with acid but no gas production

**Parvobacteriaceae.** This family, as the name implies, is composed of relatively small bacteria. The members are motile or nonmotile, gram-negative rods which in general grow most readily in media enriched with body fluids and are not active fermenters, producing acid without gas. The various species are parasitic on man and other animals. Four tribes and ten genera are recognized as follows:

Tribe	Genus	Major characteristics
<i>Most Species Can Grow on Plain Agar</i>		
Pasteurelleae .....		Fermentative, bipolar-staining rods
	<i>Pasteurella</i> .....	Milk not coagulated
	<i>Malleomyces</i> .....	Milk slowly coagulated
	<i>Actinobacillus</i> .....	Milk generally unchanged
Brucelleae .....		Nonfermentative rods
	<i>Brucella</i> .....	Parasitic for a number of animals and man
Bacteroidae .....		Anaerobic
	<i>Bacteroides</i> .....	Cells have rounded ends
	<i>Fusobacterium</i> .....	Cells have pointed ends

*Most Species Require Blood or Other Vitamin-containing Supplements to Plain Agar for Growth*

Hemophileae .....		On first isolation growth is promoted by the addition of blood
	<i>Hemophilus</i> .....	Nonmotile cells occurring singly
	<i>Moraxella</i> .....	Nonmotile diplobacilli
	<i>Noguchia</i> .....	Motile, encapsulated cells
	<i>Dialister</i> .....	Nonmotile, anaerobic organisms

**Bacteriaceae.** A considerable number of bacteria with the main characteristics of the Eubacteriaceae do not fit into any of the 11 families mentioned in the preceding discussion. These are all rod-shaped, asporogenous cells which may or may not be motile and whose metabolism appears to be rather complex. In many instances they have not been studied thoroughly. They are all grouped together in one genus, *Bacterium*, in the family Bacteriaceae. Possibly this family is best described as the wastebasket or catchall for species which do not appear to fit into any of the 12 other families. Other genera have been proposed to include bacteria related to each other by one or more characteristics, and these names or subgenera are scattered through the Bergey classification in appendixes as follows:

<i>Achromobacter</i> and <i>Flavobacterium</i>	Gram-positive, motile bacteria formerly classed in <i>Achromobacteriaceae</i>
<i>Cellamonas</i>	Small, gram-negative rods capable of fermenting cellulose
<i>Saccharobacterium</i>	Variable, gram-negative rods capable of utilizing complex polysaccharides for growth
<i>Agarbacterium</i>	Gram-negative, agar-digesting bacteria
<i>Methanobacterium</i>	Anaerobic, usually gram-negative rods capable of reducing carbon dioxide to methane

Classification of this heterogenous collection of species is extremely difficult, and the family *Bacteriaceae* should be regarded simply as a makeshift arrangement to provide a place for listing those organisms which so far do not fit into the present scheme.

**Bacillaceae.** This family contains the rod-shaped bacteria capable of producing endospores. Both physiological and morphological characteristics (in particular shape and location of spore in sporangium and bulging of the latter) are employed in the establishment of species. The majority of the bacteria in this family are gram-positive, saprophytic cells commonly found in the soil. The family *Bacillaceae* is divided into two genera as follows:

<i>Genus</i>	<i>Major characteristics</i>
<i>Bacillus</i>	Aerobic, catalase producers
<i>Clostridium</i>	Anaerobic or microaerophilic, catalase negative

### PRACTICAL USE OF THE BERGEY SYSTEM

The various genera and families of the *Eubacteriineae* have been presented in the preceding pages in outline form. Let us now consider that a bacterium has been isolated in pure culture from fresh sewage and that we wish to identify it. Assume that its general characteristics are those of the *Eubacteriineae* and that its morphology, staining properties, growth requirements, and biochemical reactions have been determined in the laboratory. It is a short, colorless rod approximately 0.5 by 2.0  $\mu$ , gram-negative, motile (apparently by means of peritrichous flagella), does not form endospores, and will grow in a medium devoid of organic nitrogenous matter, but it does require at least one organic compound as a source of carbon. It is a facultative aerobe and ferments a considerable number of sugars with the production of acid and gas. First of all, to what family does it belong?

Since it requires at least one organic compound for growth, *Nitrobacteriaceae* is ruled out. Absence of spore formation eliminates the *Bacillaceae*. Type of flagellation and general morphology eliminate families II and III. The production of acid and gas from carbohydrates elimi-

notes the Rhizobiaceae and the Achromobacteriaceae, the rod shape the Micrococcaceae and Neisseriaceae, and gram negativity the Lactobacteriaceae and Corynebacteriaceae. The organism is more active fermentatively than the Parvobacteriaceae, and the various observations taken together suggest that it is a species of Enterobacteriaceae.

The organism ferments lactose with the production of acid and gas, thus practically eliminating the tribes Proteae and Salmonelleae. Failure to produce pigment tends to rule out the Serrateae, and the search for the identification of the species narrows to the possibility of the organism being a member of the tribes Eschericheae or Erwineae. The tribe Erwineae is not a serious contender for the organism, which was isolated from sewage, but plant pathogens do gain entrance to such an environment and must be considered. The source, however, is suggestive of Eschericheae, and an attempt can be made to determine whether or not the unknown organism is a member of this tribe.

The genus *Klebsiella* can be eliminated because the unknown is motile, and there was no evidence that a capsule was produced. This narrows the identification to the genera *Aerobacter* and *Escherichia*. Differential tests for these genera can then be carried out. On finding that the organisms produced sufficient acid in glucose broth to develop the acidic color of the indicator methyl red and that acetylmethylcarbinol was not produced, the unknown could be assigned to the genus *Escherichia*. Additional differential tests could then be carried out for the determination of the species according to the criteria for differentiation as listed in Bergey's Manual. Identification is not always as easy as this example indicates, but the example does suggest that the system is of considerable value as an aid in the determination of the identity of an unknown organism. For this reason alone, the system is extremely valuable, and furthermore it is an attempt to develop possible relationships between the various groups of bacteria. Further studies will aid in untangling phylogenetic and other biological relationships between the bacteria.





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